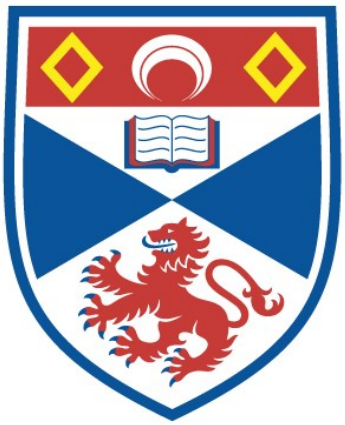
AN INVESTIGATION OF COLIFORMS AND GROUP D STREPTOCOCCI FROM ABOVE AND BELOW A SEWER OUTFALL AND THE INCIDENCE OF ANTIBACTERIAL AGENT RESISTANCE AMONGST SUCH ISOLATES

Mensah Blankson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1981

AN INVESTIGATION OF COLIFORMS AND GROUP D STREPTOCOCCI FROM ABOVE AND BELOW A SEWER OUTFALL AND THE INCIDENCE OF ANTIBACTERIAL AGENTS RESISTANCE AMONGST SUCH ISOLATES.

being a thesis presented by

MENSAH BLANKSON

to the University of St. Andrews in application for the degree of Doctor of Philosophy.

September, 1981.

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, University of St. Andrews, under the joint supervision of Dr. S. Bayne and Dr. D. Thirkell.

.....

Mensah Blankson

CERTIFICATE

We hereby certify that Mensah Blankson has spent 12 terms engaged in research work under our direction, and that he has fulfilled the conditions of Ordinance General No.12 of the University of St. Andrews and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

..... Senior Lecturer

..... Senior Lecturer

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors Dr. S. Bayne and Dr. D. Thirkell for their sustained encouragement, guidance and advice throughout the course of this work. Thanks to the University of St. Andrews for the award of the Maitland Ramsay Scholarship. My thanks are also due to Dr. J.D. Barrie for helpful discussions.Dr. J. R. Kinghorn for his interest and encouragement in the plasmid isolation, Dr. P. Montague for electron microscopy, to Mrs. M. Irvine for typing the manuscript and to everyone who helped in any way.

I would also like to thank Mr. J. Newton for help with statistical methods.

Finally, I would like to thank Burroughs Wellcome for samples of trimethoprim lactate and sulphamethoxazole, Beecham Research Labs., for ampicillin and Lilly Research Center Ltd., for cephalexin.

DEDICATION

This thesis is dedicated to my wife, Caroline Daba, and to my children, Songo Manuela, Akoto Kweku and Densuah Carmen.

CONTENTS

LIST OF TABLES

FIGURE LEGENDS

ABSTRACT

The numbers and species of coliforms and group D streptococci isolated from water samples taken from above and below a sewer outfall by the membrane filtration method were compared. In addition, group D streptococci obtained from a variety of clinical sources were also speciated.

The numbers of coliforms below the outfall were shown to increase by a factor of 36, whereas group D streptococci below increased by a factor of 150, as compared with the counts above.

Speciation of the isolates indicated that Escherichia coli and Enterobacter species were the most common coliform species from both sampling sites. Streptococcus faecalis strains were the most prevalent group D streptococci from below the outfall and from clinical sources whereas Streptococcus faecium var casseliflavus strains predominated above.

The determination of the Faecal Coliform (FC) to Faecal Streptococci (FS) ratios indicated that pollution in both sites was mainly from human origin.

The "in vitro" susceptibility of all isolates to antibacterial agents was tested by the agar dilution method. Chloramphenicol and trimethoprim were most active against all coliform isolates, followed by tetracycline, cephalexin, sulphamethoxazole, ampicillin and streptomycin in that order. Drug resistant coliforms were encountered from both sampling sites, and a significant number of multiple drug-resistant coliforms, particularly E. coli were detected. Both E. coli and Enterobacter species from below the outfall showed a statistically significant increase in resistance to ampicillin, and E. coli strains from below the outfall also showed a

statistically significant increase in resistance to sulphamethoxazole as compared with isolates from above. Ampicillin and penicillin were the most active drugs against all species of group D streptococci. Of the two aminoglycosides tested gentamicin was more active than streptomycin. Erythromycin was highly active against more than half of the strains. Tetracycline resistance was most frequent followed by Streptomycin. Streptococcus bovis and Streptococcus equinus strains were, in general, more susceptible to the drugs tested than were the other species. Streptococcus faecium strains displayed the widest range of resistance to the drugs tested. No multiple-drug-resistant group D streptococci species were encountered above the outfall, but a few isolates from below and from clinical sources were multiple-drug-resistant. Statistical analysis showed no significant increase in drug resistance between isolates from above as compared with isolates from below.

Conjugation studies indicated that for both groups of organisms, the drug resistance markers were transferable. Curing experiments with acridine orange showed a very low capacity of the agent to eliminate the R-factors from these bacteria.

1. INTRODUCTION

1. INTRODUCTION

1.1. General Considerations

Water, being one of the most important compounds on this planet, maintains life and its processes and throughout recorded history it has been known in a qualitative way, that it is also a medium in which human diseases can be transmitted. Yet, its role in disease transmission was only discovered in the second half of the 19th century by the pioneers of the study of water-borne bacterial diseases, beginning with the classical investigations of John Snow (1855) on the spread of cholera by water, and those of William Budd (1856; 1873) on water-borne epidemics of typhoid fever. As a result, initially from health consideration, but latterly from the need to maintain an acceptable environmental quality, a controlled approach to waste collection and disposal was developed. For many years, and especially over the past sixty years, groups of workers from widely differing scientific disciplines, have succeeded in developing methods, mainly by empirical means, for the detection of the micro-organisms responsible for water-borne diseases. Consequently, diseases such as cholera and typhoid fever have almost been eradicated from countries where adequate sewage treatment and sanitary control of water supplies is maintained. Annual mortality rates from typhoid fever as an example, have dropped in the United Kingdom since 1900, from about one in a hundred to less than one in 54 million and indeed, during 1980 only one patient with typhoid fever died (Hospital Doctor, 1981).

In the United Kingdom where over 95% of the population are supplied with piped drinking water, almost all of it chlorinated, (D.H.S.S.(Welsh Office), 1969), outbreaks of water-borne bacterial diseases are rare. The occasional outbreaks(Green *et al.* 1968),

are attributed almost always to accident or to the failure in application of known control measures rather than to the lack of needed knowledge. But in the developing countries where in 1970 only 10% of the rural and 50% of the urban population were supplied with safe drinking water (Wilson and Miles, 1975) and where materials and techniques required for effective sewage treatment are not readily available, outbreaks of water-borne microbial diseases are in epidemic proportions (W.H.O., 1974).

The preventive measures for the control of such outbreaks can be grouped into three broad categories:

(i) the protection of water supplies from initial contamination

(ii) the removal of pathogenic micro-organisms from water supplies by filtration, adsorption or similar mechanisms and,

(iii) chemical destruction or inactivation of the micro-organisms by the process of disinfection.

The primary objectives of these measures are to alleviate health hazards and to reduce the concentration of oxidizable organic compounds, yielding a final product which could be discharged into the natural environment without producing adverse effects. Water supplies have been adequately protected from bacterial contamination by any one or all of these measures. However, these approaches have been subjected to continually greater stress by rapid urbanization, constantly increasing population density and the demand for water for consumption and recreational use. Hence, it is increasingly difficult to find relatively uncontaminated sources of water as the demand grows and as the extent of contamination from human, animal and industrial wastes increases and spreads. At first these measures were designed to deal with domestic wastes, since such wastes have a relatively standard composition and contain readily degradable materials. Since the turn of the century,

however, the types of wastes produced have altered radically due to advances and expansion of modern industrial processes and agricultural practices. Therefore, physical and chemical treatment of water supplies must be constantly improved to maintain hygienic quality and constantly monitored to detect microbial contamination.

The early methods of water purification were directed against the enteric pathogenic bacteria such as those responsible for cholera, dysentery and typhoid fever. However, it has become apparent in recent years that these procedures are not adequate for the control of those viral diseases which under certain conditions may be water-borne. Indeed, it is only recently that infectious hepatitis was added to the list of water-borne microbial diseases (Berg, 1966). Epidemics of infectious hepatitis are now known to have been caused by water contamination, the most dramatic of which occurred in New Delhi, India, in 1956 when a total of nearly 30,000 icteric cases was reported (Berg, 1966). Contaminated water may in addition contain other viruses of the Entero-virus group which are now known to be water-borne but they have not been shown definitely to be involved in the transmission of disease by this route (Rheinheimer, 1974). With the exception of the poliomyelitis virus, there is yet to be found some confirmatory epidemiological patterns. However, there appears to be some basis for the belief that sporadic outbreaks of mild diarrhoeas and respiratory disorders may be caused by viruses. In addition to the viral diseases, certain disorders caused by Legionella and Campylobacter, whose aetiology was until recently inexplicable, are now thought to be transmitted via the water route.

The factors in the spread of water-borne diseases from person to person and from community to community are not different from those involved in their spread internationally. Quarantine measures only apply to controlled passenger traffic particularly in the developed countries. In the underdeveloped countries, infection is spread through uncontrolled migration (W.H.O., 1967). Widespread primary immunization especially in the poor countries is necessary, but emphasis should not be placed on immunization alone.

In accordance with the accepted principles of community water supplies (W.H.O., 1959), "every effort should be made to provide conveniently distributed safe water in ample quantities to meet all personal and household needs essential for sanitation and cleanliness."

Acceptable standards for safe water quality have been proposed (W.H.O., 1961; 1963; D.H.S.S.(Welsh Office), 1969; 80/778/EEC., 1980). The list of chemical agents that pollute water is enormous and well documented (Chenlett, 1979; Higgins and Burns, 1975). Throughout this study, the aspects of pollution considered will be limited to bacterial contamination by faecal matter of man and animals. Wherever appropriate other sources of pollution will be mentioned.

1.2. The Origin and Diversity of Bacteria found in Water

Bacteria are uniquely ubiquitous on the earth's surface because of their ready dissemination by water and wind. It is not surprising to find bacteria in natural waters except supplies from deep bore-holes. Since all waters at some period in their history have come in contact with this surface, the types of bacterial population found in any given water depend on the extent of contact and character of this surface. The preponderance of bacteria found in waters free from gross pollution originate from the air, soil or vegetation. Some of these are able to multiply and continue their normal existence whilst others are unsuccessful. Those capable of surviving in surface waters for long periods are often referred to as the "natural water bacteria". They consist mostly of cocci, fluorescent bacilli, chromogenic and non-chromogenic bacilli, the sulphur-producing bacteria and the iron-oxidizing bacteria. These bacteria are generally harmless and are of no great significance except in the blockage of water pipes by colonization and the destruction of equipment by corrosion (Round Table Discussion, Second International Symposium on Microbial Ecology, 1980).

Some soil bacteria of which the aerobic spore-bearers are examples, are washed into waters particularly in times of flood and heavy rainfall. Certain members of the coliform group of bacteria, for example, Klebsiella aerogenes and Enterobacter cloacae, whose natural habitats are in the soil, on grains, plants and decaying vegetation (Wilson and Miles, 1975), may be found in surface water without the water receiving sewage or sewage-sludge pollution. Considering the hygienic and sanitary aspect of water quality, bacteria derived from the disposal of treated or untreated domestic wastes into waters constitute the most important group. Many of the organisms in this group, for example, Escherichia coli, Streptococcus faecalis and Clostridium perfringens are the "normal" inhabitants of the intestinal tracts of man and animals. They are not found in pure waters or in virgin soils and sites remote from human and animal life (Medrek and Litsky, 1960; Holden, 1970). Others such as K. aerogenes and E. cloacae, although also found in the intestinal tracts of man and animals, show greater powers of survival and may multiply on decaying or other materials that satisfy their nutritional requirements. The majority of these organisms are quite harmless but enteric pathogens such as Vibrio cholerae, Salmonella typhi and paratyphi or Shigella dysenteriae may be present; and of the two sources man is by far the more dangerous. Bacteria derived from sewage are foreign to water and most of them tend to die out gradually.

1.3. Factors that influence the survival of faecal bacteria in water

The need for better understanding of the factors that affect the survival of water-borne pathogenic bacteria in water, their mechanisms of penetration and spread through soil into ground water, and their sensitivities to methods of water treatment has led to several sophisticated studies with conflicting results. This knowledge is required in assessing the suitability of water for consumption and recreational use and for the sanitary survey of rivers and streams.

Water plays host to a vast array of microorganisms from fungi to protozoans and bacteria to viruses. The bacterial population density of a given surface water is subject to wide variations and it changes from day to day. Faecal bacteria are foreign to water and only the better adapted groups can survive for long periods. Pathogenic organisms, generally, have a low survival rate as compared to the "normal" intestinal bacteria. Bacteria, as with other forms of life, are subjected to the rule of "survival of the fittest" and in order to survive they must overcome hostile factors. The major factors are:

(i) availability of nutrients
(ii) pH
(iii) temperature
(iv) sunlight
(v) dissolved oxygen and
(vi) the presence of other microorganisms.

1.3.1. Availability of Nutrients

Bacteria have quite simple nutritional requirements and, as an example, pure deep well water can support comparatively large numbers of bacteria. Under certain conditions bacteria may live for long periods in distilled water (Bigger and Nelson, 1941; 1943). Allen et al.,(1952) observed that E. coli required very little organic matter to survive in a buffer solution whereas S. faecalis required a much higher concentration. Evison and James (1975) asserted that nutrient concentration would enhance the survival of E. coli compared with the faecal streptococci since the nutrient concentration required for regrowth of E. coli is considerably less than that for faecal streptococci. This observation may account for the high concentration of nutrient required by S. faecalis as observed by Allen and his collaborators (1952). The nature and quality of organic compounds present in any given water which can be utilized as carbon and energy source reflects the number of different strains present

and also the species that develop. When organic compounds are in abundance the bacterial population density increases. Conversely, when it is low or scarce they are few and tend to die out and the viable varieties are probably due to differences in phenotypic responses to the fluctuating organic substrate and to the physical conditions in the micro-environment of the bacterial cells (Konings and Veldkamp, 1980).

1.3. 2. pH

Another factor affecting the survival of bacteria in water is the pH. Only a few groups of bacteria can tolerate any substantial degree of shift from their optimum pH. Both acidity and alkalinity are inimical to the growth of most groups of bacteria. The acid tolerance of bacteria varies with species; the aerobic and anaerobic spore-bearing bacteria are usually acid-resistant as also the strictly anaerobic bacteria. McIntosh et al. (1922) reported the unusual resistant nature of lactobacilli to acid. The limiting pH for survival for a majority of bacteria lies between the extremes of pH 4 and pH 9. Geldreich and Kenner (1969) have suggested pH values within the limits of pH 4-9 as suitable for the application of the Faecal Coliform (FC) to Faecal Streptococci (FS) ratios. For E. coli the optimum pH for growth is pH 7.6 and the optimum for survival is pH 6.0 (Wilson and Miles, 1975). Allen et al. (1952), on the other hand, reported a pH range of pH 6-7 as the range in which both E. coli and S. faecalis were least viable. Just as bacteria have a limit of acid-tolerance so they possess a limit of alkali-tolerance. Cohen (1922) found that for Salmonella typhi this was about pH 8.7 and H^+-ions concentration appear to be more toxic than OH^--ions. S. faecalis, Streptococcus faecium and Vibrio cholera have a high alkali tolerance and can grow in artificial medium at pH 9.6.

1.3.3. Temperature

Temperature, like pH, has a marked influence on the survival of bacteria in water. The response of different bacteria to temperature

changes varies greatly. Lane-Claypton (1909) showed that the generation time of E. coli in broth was 0.32 hr at 42°C and 1.3 hr at 20°C. Other reports suggest an increase in numbers of coliforms and E. coli in raw water stored up to 3 days at room temperature (Pub. Health Lab. Sci. Water Subcommittee, 1952; Coles and Simpson, 1958). Studies on the survival of S. typhi were conducted as early as 1889 when the organism was reported to survive in soil for 5.5 months (Grancher and Deschamps, 1889) and for 3 months (Karlinski, 1889). Houston (1913) showed that low temperature favoured the survival of S. typhi in raw river water. Hamilton (1935) noted the reduction in numbers of colon bacteria by 16% in winter months and by no less than 97.97% in the summer months. Davenport et al., (1976) also noted that on ice-covered water 15.5% faecal coliforms survived after 7.1 days as compared with 32.8% of faecal streptococci. On the other hand, studies on soil samples showed that faecal coliforms survive slightly longer than faecal streptococci during the summer, but in winter and spring the faecal streptococci survive much longer than faecal coliforms (Van Donsel et al. 1967). During the warmer temperature of the summer months, sewage bacteria multiply rapidly with a significant increase in their numbers within 24 hr. This may be due to the amount of utilizable organic substances present. The freezing weather of the winter months independent of the utilizable organic substances present arrests the activities of water bacteria rather than kills them. A sudden change in temperature-thermal shock may destroy bacteria that survive when the changes are made slowly and may also account for the rapid decline in the numbers of intestinal bacteria when discharged into the natural environment.

1.3.4. Bactericidal Effects of Sunlight

The ultra-violet rays of sunlight may play a part in the destruction of organisms in river waters. Perhaps the most important spectral region is that around 260nm to 300nm wavelength both from photobiological and

ecological points of view. Towards 300nm important processes such as photosynthesis, photoreactivation and phototaxis take place and below 300nm wavelength both proteins and nucleic acids show increased absorption. Gates (1930) in his study of the relative effectiveness of radiation of different wavelengths on the survival of coliform bacteria showed that for E. coli 265nm was most lethal. Similar studies (Rupert, 1964), also showed that the ultra-violet light contained in natural sunlight caused damage to DNA. Although the ultra-violet rays of sunlight may, therefore, play a part in the destruction of organisms in shallow waters especially clear waters, but even in very clear waters the movement of water cuts down the exposure time of any given bacterium to the ultra-violet rays; moreover, ultra-violet rays do not penetrate for more than 5ft below the surface (Wilson and Miles, 1975). Sunlight plays a relatively unimportant part in the destruction of organisms in the United Kingdom and is also ineffective in turbid waters. However, a recent report indicates that the visible rather than the ultra-violet light spectrum of the sunlight is responsible for bactericidal effect in diluted sea water (Fujioka et al. 1981).

1.3.5. Dissolved Oxygen

Whereas dissolved oxygen, measured in terms of Biological Oxygen Demand (BOD), is favourable for the survival of aerobic and facultative anaerobic bacteria in water, it is not so for the strict anaerobes. Whipple and Mayer (1906) observed that S. typhi remained viable in sterile water containing dissolved oxygen longer than in water kept under anaerobic conditions. In another experiment S. typhi survived for nearly 2 months in filtered tap water exposed to air at room temperature and died within 4 days in an atmosphere of hydrogen. Thus, they suggested that this may partly explain why S. typhi dies more rapidly in polluted water than in pure water and also why this organism survives for a shorter time

in the summer than in winter. However, Taylor (1940), on the contrary, failed to demonstrate any close relationship between the amount of dissolved oxygen in lake water and the bacterial content.

1.3.6. Microbial Interactions

It has been known for a long time that waters from some rivers are relatively lethal to certain intestinal bacteria. The destruction of the bacteria in such cases is more rapid than can be explained by the processes of self-purification. But it was not until the early 20th century that the Twort-d'Herelle phenomenon, bacteriophage, was proposed which provided an excellent explanation of this bactericidal property of river waters. Twort (1915) noted a peculiar degenerative change in cultures of staphylococci which by a process of autolysis disrupted the culture leaving a granular debris. In 1917 d'Herelle independently recorded similar findings with dysentery bacteria. Bacteriophages are now known to be associated with most bacterial families. Phages for a given bacterial species can often be isolated wherever that species occurs in nature. For example, phages for intestinal bacteria are found in faeces, sewage and polluted water (d'Herelle, 1921; Guelin, 1952); and phages for Bacillus species in sewage or soil, (Cowles, 1931; Iantos et al., 1960). They may also be isolated from lysogenic strains of the same or related species. Spencer (1963) observed that phages active against a variety of Enterobacteriaceae can readily be demonstrated in polluted waters but not in rivers remote from terrestrial contamination. Rheinheimer (1974) is also of the opinion that phages play a part in the rapid decrease in bacterial numbers in sewage polluted water. Thus, phages play some part in the self-purification of polluted water which may account for the great diversity of findings with regard to the survival of intestinal organisms in water, and may also account for the more rapid die-off rates of pathogenic bacteria in polluted water than in pure ones.

Both ciliate and flagellate protozoa are also known to feed on bacteria and are involved in sewage treatment processes.

1.3.7. Comparative Survival of Indicator Bacteria and Enteric Pathogens

Comparative survival studies on indicator organism and enteric pathogens indicate that enteric pathogens die-off more rapidly than coliforms in well water (McFeters *et al.*, 1974). Similar studies in sea water indicate the opposite, that pathogens survive longer than *E. coli*. (Round Table Discussion, Second International Symposium on Microbial Ecology, 1980).

1.4. Why Examine Water for Bacteria?

The reason for the bacteriological examination of water is to determine the degree of contamination of water with wastes from human or animal sources. These waters include water suitable for domestic, industrial and recreational use. With the increase in use of natural waters for receiving treated effluents and the disposal of sewage sludge on to agricultural land, it is necessary to monitor the sanitary and hygienic quality of water. Traditionally, tests for the detection and enumeration of the indicator organisms, rather than that of pathogens have been used. The reason being that if these easily detectable indicator organisms, usually harmless enough themselves, appear to be present, they may signal the presence of disease-producing organisms which have devastated mankind throughout history causing typhoid fever, dysentery, cholera and infectious hepatitis. To protect community health microbiological procedures have been developed to test for these microorganisms.

If the contamination is recent and among the contributors there are cases of carriers of enteric fever or dysentery, the water may contain living bacteria which cause the disease and the drinking of such water, and its use in the preparation of food which may encourage the multiplication of the pathogens, presents a great risk to public health (D.H.S.S. (Welsh Office), 1969). The faeces of animals and birds, particularly seagulls, may carry human intestinal organisms pathogenic to man (Hobbs, 1961; Fenlon, 1981).

The attempts of early water bacteriologists to isolate the specific pathogenic bacteria in water were generally unsuccessful. Their failure was partly due to technical and cultural difficulties. However, with improved techniques and the introduction of modern selective media greater success is now possible. For routine control purposes a direct specific search for pathogenic bacteria is impracticable and should not be the only procedure for the following reasons:

(i) the water supply rarely comes under examination immediately after the specific contamination;

(ii) the contamination may be accidental or isolated rather than continuous;

(iii) the causative organism(s) may have by then disappeared; and

(iv) the water in any case would have been consumed.

Moreover, pathogenic bacteria, if present in water are usually greatly outnumbered by the "normal" intestinal organisms which tend to survive longer than the majority of pathogens. Also copious volumes of water may need to be examined, and selective media are required for their isolation and their identification involves a battery of biochemical and serological tests (D.H.S.S.(Welsh Office), 1969).

For these reasons, the routine bacteriological examination of water is concerned with the detection of those bacteria that are the harmless inhabitants of the human and animal intestinal tract, as these will reveal the presence of organisms excreted by humans and warm-blooded animals. These faecal bacteria are constantly present, usually in large numbers, in the faeces of man and animals so that the pollution of water by exceedingly small traces of faeces can be detected bacteriologically. Bacteriological examination of water is, therefore, a sensitive method for the detection of faecal pollution.

The ideal indicator organism should possess several reliable characteristics which Bonde (1962) has aptly described. Buttiaux and Mossel (1961) listed the ideal requirements for an indicator organism as:

(i) specificity

(ii) occurrence in high numbers

(iii) response to extra-enteral environment and

(iv) ease and reliability of detection.

Bearing in mind that bacterial variation prevents adherence to hard and fast rules, a variety of important characterisation tests would allow for the ease of detection of these organisms, rather than a few selected tests. In the following sections, the significance of these indicator organisms, particularly the coliforms and the faecal streptococci and their occurrence in the presence or absence of pathogens, their definition and distribution will be discussed.

1.5. The Normal Intestinal Flora of Man and Animals

The organisms generally used as indicators of faecal contamination of water are those which are normally present in large numbers in the human and animal intestine. Although the normal human intestinal flora had been studied as far back as 1919, yet it was only over 20 years ago that serious attention was paid to the microbiology of animal intestinal contents (Briggs *et al.* 1954; Willssens and Buttiaux, 1958; Cooper and Ramadan, 1955; Haenel, 1960; Smith and Crabb, 1961; Dickinson and Mocquot, 1961; Raibaud *et al.* 1961; Geldreich and Kenner, 1969).

1.5.1. Human Intestinal Flora

The human intestine contains many species of commensal bacteria with two types of micro-flora, the resident flora which varies very little under constant conditions, and the transient flora, which is introduced with meals and causes a wave-like increase in the microbial population. Although a "normal" flora in one community may be

abnormal in another consuming a different diet or exposed to a different climate, certain microorganisms are adapted to certain sites of the human body and have entrenched themselves so firmly that it is extremely difficult to dislodge them except by drastic means. Thus E. coli appears to be a normal intestinal parasite of man in all parts of the inhabited globe (Wilson and Miles, 1975).

Anaerobic bacteria constitute the majority of the faecal flora and make up approximately 98 to 99% of the total bacterial population. Tabaqchali, (1974)(Table 1.1) listed the main groups of bacteria and the types commonly isolated together with their concentrations per gram of faeces. Phillips,(1981) (Table 1.2)listed the normal anaerobic flora of man according to their prevalence.

Bacteroidesand Bifidobacteria are the predominant anaerobic groups. The aerobic flora is represented by the Enterobacteria with E. coli being the predominant aerobe and by Streptococcus faecalis and aerobic lactobacilli. The normal flora of man is well documented in Skinner and Carr (1974); Drasar and Hill (1974); for a review see Savage (1977).

TABLE 1.1 BACTERIA OCCURRING IN THE INTESTINE AFTER TABAQCHALI (1974).

Major bacterial groups	Counts/g wet wt. faeces	Species often isolated
Enterobacteria	10^7 - 10^8	*Escherichia coli*
		Klebsiella aerogenes
		Proteus mirabilis
Streptococcus	10^6 - 10^7	*Streptococcus viridans*
		Streptococcus faecalis
Lactobacillus	10^5 - 10^6	*Lactobacillus acidophilus*
		Lactobacillus casei
Clostridium	10^4	*Clostridium perfringens*
		Clostridium sporogenes
Veillonella	10^7 - 10^8	*Veillonella parvula*
		Veillonella alcalescens
Bacteroides	10^{10} - 10^{11}	*Bacteroides fragilis*
		Bacteroides melaninogenicus
Bifidobacterium	10^{10} - 10^{11}	*Bifidobacterium adolescentis*
Eubacterium	10^{10} - 10^{11}	*Eubacterium biforme*

TABLE 1.2. THE NORMAL ANAEROBIC FLORA OF MAN AFTER PHILLIPS (1981)

Anaerobic bacteria	Prevalence of anaerobes in flora of			
	Skin	Upper respiratory tract	Vagina	Large intestine
Gram-positive cocci				
Peptococcus				
Peptostreptoccus	+	++	++	++
Gram-negative cocci				
Veillonella		++	++	+
Gram-positive bacilli				
Actinomyces		+		
Bifidobacterium		+	+.	++
Clostridium				++
Eubacterium		+		++
Propionibacterium	++			
Gram-negative bacilli				
Bacteroides fragilis				++
Other Bacteroides species		++	+	++
Fusobacterium		++		+
Spirochaetes				

1.5.2. Animal Intestinal Flora

Like the human intestinal flora, most of the individual members of the animal flora belong to the anaerobic groups Bacteroides, Bifidobacterium and Lactobacillus (Mossel, 1959). Amongst the aerobic flora faecal streptococci usually out-number E. coli and depending on the host, the predominant streptococcal species differs. The species distribution of streptococci is discussed elsewhere in the text.

1.6. The Bacterial Indicator Organisms

1.6.1. The Coliform Group of Bacteria

The organisms most widely used to indicate the degree of contamination of water with wastes from human or animal sources are the "coliform" group as a whole and E. coli in particular. The term "coliform bacteria" as defined by Wilson and Miles, (1975), refers to those enterobacteria, other than Salmonella, Shigella and Proteus, that generally, though by no means always, ferment lactose. The application of the name coliform is deeply rooted in water bacteriology. When coliform is used in this study it will be in the most restricted sense conforming to the definition given by Wilson and Miles (1975). The distinction of lactose fermentation is not infallible as various strains of coliforms, particularly E. coli, that have temporarily or permanently lost their ability to ferment lactose due to stress or injury, have been described (Round Table Discussion, Second International Symposium on Microbial Ecology). Definitions

other than that given by Wilson and Miles are abundant in the literature and are not necessarily in agreement. Mackie (1913) put forward the view that lactose fermentation is no more important than any other sugar reaction in the differentiation of the coliforms. Mossel (1957) shared this view and recommended the replacement of lactose with mannitol. But this view is not generally accepted and lactose still remains the sugar of choice in most primary isolation media.

1.6.1. (a) The Classification of the Coliform Group of Bacteria

The coliform bacteria belong to the family Enterobacteriaceae. Pathogenic species such as S. typhi and Shigella dysenteriae and other species which are opportunistic pathogens such as Citrobacter freundii, Enterobacter cloacae, E. coli, K. aerogenes and Serratia marcescens are also found in this group. This is the principal reason for the interest which water bacteriologists take in this group. All members of the family are gram-negative non-sporing rods, aerobic and facultatively anaerobic, catalase-positive, oxidase-negative, nitrate reduced to nitrite, often motile and attack sugars fermentatively (Cowan, 1974). These organisms have been studied for many years by groups of workers with widely differing objectives. Whereas the early water bacteriologists were concerned in distinguishing the organisms found in the intestine from those living saprophytically outside the animal body, the medical and veterinary bacteriologists concentrated their attention on those organisms which cause illness in man and animals. Consequently a great deal of confusion arose due to this diversity of approach as the same organism was given different names by different workers. Recently, and largely due to the efforts of the Enterobacteriaceae Sub-committee of the International Association of Microbiological Societies, a general agreement on the definition of this group has been reached (Wilson and Miles, 1975).

A large number of tests have been devised with the object of

separating this group into its constituent members, and generally the greater the number of tests used the greater the number of species and strains differentiated. For the purpose of practical water bacteriology a comparatively small number of tests is recommended (D.H.S.S.(Welsh Office), 1969).

These tests are the production of acid and gas from lactose at 44°C, together with indole production (I) and methyl-red (M), Voges-Proskauer (V) and citrate utilization (C) as sole carbon source, tests. The results of these tests can be expressed by plus and minus using the acronym IMViC (Parr, 1938). However, IMViC typing presents some problems since these tests only serve to differentiate coliform organisms into biotypes. Closs and Digranes (1971), have shown that IMViC patterns are non-specific relative to the genera of the coliform group. The ambiguity caused by IMViC typing is most evident with the genera Klebsiella and Enterobacter, both of which have the --++ IMViC pattern.

To determine the meaningful relationship between sources of pollution and potential health hazards, laborious and time-consuming ancillary biochemical procedures are necessary. Using a carefully selected reproducible range of biochemical tests, a differentiation of members of this group can be obtained which is of much more value than results obtained from IMViC biotyping reactions. From the results of extensive work by several workers, tests for characterizing members of this group are documented (Bergey, 1974; Cowan, 1974; Wilson and Miles, 1975).

1.6.1. (b) <u>E. coli: Is too much significance attached to its occurrence in water?</u>

Owing to the early pioneering work of Houston (1913), <u>E. coli</u> came to be regarded as the most suitable indicator organism of faecal pollution in the United Kingdom (Wilson and Miles, 1975). This is largely due to its relation to the salmonellae and was found to occur in much greater numbers than these organisms in faeces. Since then, little attention

has been focussed on the other members of the coliform group and doubts have been cast on the validity of E. coli as the sole indicator organism of faecal pollution.

Although E. coli counts have survived the tests of time, the search for and the enumeration of this organism alone in water must not be taken as the criterion of water quality. The limitation to the demonstration of E. coli in water samples is that the source of contamination whether from human, animals or birds cannot be distinguished.

Instances have been reported where the findings of pathogenic organisms have not been associated with the detection of E. coli. In acute intestinal disease and in the carrier state, the "normal" inhabitants of the intestine may be largely replaced by pathogenic organisms (Thomson, 1955). Seligmann and Reitler,(1965) revealed the presence of Salmonella montevideo accompanied in some cases by low or no E. coli counts during the examination of well waters in Israel where sporadic cases of enteritis occurred. Grunnet and Nielsen, (1969) also demonstrated the presence of Salmonella species at various sampling points in the Bay of Aarhus, Denmark, where E. coli was absent.

1.6.1.(c) Are Other Members of the Coliform Group Significant?

The significance of the presence in water of other members of the coliform group has been much debated by Thresh et al., (1958). Bardsley (1934) expressed the opinion that the presence of coliforms other than E. coli in water samples denoted distant or remote association with faecal pollution. Several objections have been put forward against the use of a total coliform count as an indicator of water pollution. The objections (Wilson and Miles, 1975) are:

(i) not all species originate from faecal sources

(ii) these organisms gain access adventitiously to the human body with food

(iii) they are not commensals of the intestine, persist in it for only

a short time, and are excreted in faeces, as a rule in much smaller numbers than E. coli.

However, the presence of E. coli in polluted waters is usually associated with the presence of these coliforms and they are also always present in human excrement. As a result, they are now generally accepted as alternative indicator organisms.

In recent years, pathogens have been isolated from waters which, based on coliform standards, should have been safe. For instance, Muller (1964) isolated Salmonella from coliform-free water during the Hamburg flood of that year. Fair and Morrison (1967) also isolated Salmonella from waters containing only 30 coliform organisms per 100ml. Gallagher and Spino (1968) showed little apparent correlation between levels of total or faecal coliforms and the isolation of Salmonella. It has recently been proposed that the coliform group of bacteria are a very poor indicator of hazardous microbiological pollution of treated waters (Dutka, 1973). The need for a test organism other than the coliform group for the detection of faecal contamination resurrected the almost forgotten faecal streptococci.

1.6.2. The Faecal Streptococci

The association of streptococci with the faeces of warm-blooded animals and with sewage-polluted water has been recognised since the early 1900's (Houston, 1900; Winslow and Hunnewell, 1902). Winslow and Palmer (1910) noted that the use of these streptococci might assist in differentiating between pollution from human origin and other animal pollution. That little interest was shown in the use of those organisms as indicators of water pollution until recent years, was largely due to the lack of satisfactory cultural methods. During the 1940's and the 1950's there was a resurgence of interest in the use of these organisms as indicators of pollution. Stimulated by the improvement in cultural techniques, beginning with the introduction of the azide broth of Mallmann (1940), the modifications of Hajna and Perry (1943), Winter and

Sandholzer (1946) and Litsky et al.,(1953), and the glucose-azide broth of Hannay and Norton (1947) and Mallmann and Seligmann (1950). The glucose-azide agar of Slanetz et al.,(1955), Slanetz and Bartley (1957), and the KF streptococci agar of Kenner et al.,(1961) were employed in the membrane filtration technique which provided higher counts of streptococci associated with faecal polluted water. Hartman and his collaborators (1966) and Pavlova et al.,(1972) have provided excellent reviews of media used for the selective isolation of this group of organisms. Recently, occasional new media have appeared in the literature (Abshire, 1977; Donnelly and Hartman, 1978).

As the use of coliforms as indicator organisms came under attack, these streptococci became potentially more valuable indicators. But they have not as yet been generally accepted as indicator organisms, in their own right, of faecal pollution for two reasons. Firstly, coliform organisms have been a more attractive means of identifying faecal contamination because early workers found them easier to quantify and they are present in greater numbers in faeces, sewage and polluted water. Secondly, there is a great deal of confusion concerning the identity of the streptococci associated with faeces, particularly as it relates to their ecological distribution. A brief survey of the difficulties of characterising and sub-dividing these streptococci is presented below.

Because of their distribution and importance in water and the food and dairy industries, where their presence can be correlated with faecal contamination, a great deal of work has been done in their characterisation. Even though they have become one of the most studied and better characterised groups ambiguities still remain. This is because there is confusion about the terminology used to describe divisions within this group. Thiercelin (1899) established the taxon "enterococci" in order to place taxonomically the gram-positive 'diplococcus' present

in the faeces of humans and warm-blooded animals. The "enterococci" were characterized by morphological features, cultural characteristics and source of isolation. As more became known of their biochemical, physiological and antigenic properties and their pathogenicity for man and animals other designations were introduced; "faecal streptococci", "Group D streptococci" and even "Streptococcus faecalis" have been used interchangeably and by implication synonymously (Jones, 1978). Hartman et al. (1966) in their review of the taxonomy of the faecal streptococci, have discussed the terminology admirably. Comprehensive reviews of earlier studies may be found in the papers of Orla-Jensen (1919); Dible (1921); Sherman (1937); Frost and Engelbrecht (1940); Skadhauge (1950); Seeleman (1954); Shattock (1955), (1962); and Deibel (1964). It is now evident that these terms are not synonymous.

1.6.2. (a) Definition of the Enterococci

Those micro-organisms classified as the 'enterococci' are spherical, gram-positive cells that are slightly elongated along the axis of adherent cells. The cells are arranged in pairs or short chains with a cell diameter of 0.5-1.0μm. They grow readily in the temperature range from 10°C up to over 40°C. Some species even growing at 50°C. They are able to tolerate a temperature of 60°C for 30 min. They grow in artificial media containing 6.5% sodium chloride, 40% bile, at pH 9.6, and reduce litmus in milk (Sherman, 1937;1938). Sherman used the term "enterococcus" to designate a group of streptococci including haemolytic, non-haemolytic and gelatin-liquifying types. He regarded the "enterococcus group" as comprising S. faecalis and its variants, liquefaciens and zymogenes, S. faecium Orla-Jensen (1919), and the S. durans of Sherman and Wing (1935; 1937). There is no dispute that the "enterococci" belong to the Lancefield's group D streptococci, which contains the

glycerol type of teichoic acid as the group D specific antigenic determinant. However, this antigen is not confined to Sherman's enterococci but also occurs along with other antigenic determinants in the non-enterococcal organisms S. bovis (Shattock, 1949) and S. equinus (Smith and Shattock, 1962), S. avium and also in streptococci designated serological groups R, S and T (Elliott, 1966; Windsor and Elliott, 1975).

1.6.2. (b) Definition of Faecal Streptococci

The term "faecal streptococci" as used by Geldreich and Kenner (1969) and as recommended by D.H.S.S. (Welsh Office), 1969 and by Standard Methods (1975); should be used to designate only the following named species and variants: S. faecalis, S. faecalis var. liquefaciens, S. faecalis var. zymogenes, S. faecium, S. durans, S. bovis and S. equinus. Facklam (1972) has suggested that the term "faecal streptococci" has no definite meaning and should not be used. "Faecal Streptococci", like the application of the name coliform is deeply rooted in the terminology of water bacteriology hence it is difficult to avoid using this term in a study of this kind. These streptococci normally occur in human and animal faeces. They are therefore, most likely to be found in polluted water and their presence in water should be regarded as evidence of faecal pollution. The use of the term "faecal streptococci" in the text conforms to the definition given above.

1.6.2. (c) The Significance of the Faecal Streptococci

The detection of faecal streptococci in water samples provides valuable supplementary information on the bacteriological quality of the water. Unlike the coliforms, some of the faecal streptococci are host-specific and further speciation of this group may provide valuable additional information about the source of pollution. For example, if S. bovis and S. equinus predominate this would indicate pollution from non-human warm-blooded animals. Numerous investigations have shown that high numbers of these species are associated with pollution involving

meat-processing plants, dairy wastes and feedlot and farmland run-off.

Since the survival times of S. bovis and S. equinus outside the animal body have been shown to be short (Kjellander, 1960), it follows therefore that their presence in water indicates recent pollution by animals. Because of the variable survival characteristics within the group the faecal streptococci should not be used as the sole criterion of water quality. It is customary to use other faecal indicators concurrently. (See later.).

1.6.3. Anaerobic Bacteria

Although anaerobic bacteria were first described over one hundred years ago, only a few of them - Clostridium and possibly Bifidobacterium - have achieved any importance in the bacteriological examination of water. Because the aerobic bacteria - coliforms and faecal streptococci - are more easily isolated and identified they have become important in water bacteriology. Even in man anaerobic bacteria still remain the predominant group (Table 1.1) and could possibly be a more significant indicator of faecal pollution.

That the anaerobes - especially the non-sporing anaerobes - have no significant role in water bacteriology may be due to several reasons. These are:

(i) their survival rate outside the human body may be low

(ii) their growth is often slow

(iii) their isolation and identification takes several days

(iv) testing their susceptibility to antimicrobial agents may take several days or weeks.

The past few years have seen a major re-awakening of interest (fully reviewed by Finegold, 1977), in the anaerobes due to the development and the application of improved techniques for their isolation and identification, largely in the U.S.A. by Holdeman et al., 1977.

Amongst the anaerobic bacteria, Clostridium perfringens has been the only species to find favour in water bacteriology from the very early days. This is largely due to its toxigenicity which has forced attention on this organism by its obvious pathogenic potential. Wilson (1931) stressed that Cl. perfringens is an organism of indisputably faecal origin which is of great importance for the detection of intermittent and occasional pollution. But Cl. perfringens is not exclusively of immediate intestinal origin. It is widely distributed in nature and forms spores that are highly resistant to chemical agents e.g. chlorination, and to adverse environmental conditions. D.H.S.S. (Welsh Office), (1969), considers the detection of Cl. perfringens to be a valuable supplement to that for aerobic micro-organisms, especially when it is found in the absence of coliforms. When this occurs, it is indicative of faecal contamination which had occurred at some remote date. Bonde (1962) has fully reviewed the significance of Cl. perfringens in water.

It has been suggested that bifidobacteria could perhaps be more suitable alternative indicators of faecal pollution (Gyllenberg and Niemelä, 1959; Evison and James, 1973; Resnick and Levin, 1977). These organisms are present in even higher numbers than are coliforms, faecal streptococci and clostridia in human faeces (Table 1.1) and they die-out at a slow rate when introduced into water (Evison and James, 1973). Findings on the distribution of bifidobacteria in U.K. water sources tend to confirm that they are almost exclusively of intestinal origin (Evison and James, 1973; Opara, 1978). Like the bifidobacteria, Bacteroides fragilis is also found in high numbers in faeces (Table 1.1) and is found in comparable numbers with bifidobacteria in polluted water (Opara, 1978). That the non-sporing anaerobes occur in natural waters is now well established. Much work is required to establish how well they

survive and their distribution in the natural environment.

1.7. Role of Faecal Coliform to Faecal Streptococci Ratios

In an attempt to distinguish between water pollution originating from a human or non-human source, Geldreich (1966), Geldreich et al., (1968), Geldreich and Kenner (1969) suggested that the ratio of Faecal Coliforms (FC) to Faecal Streptococci (FS) may be used to indicate the source of faecal pollution. In recent years, Mara (1974) and Feachem (1975) have put forward data to support this suggestion. The premises are (1) the different initial bacterial composition and (2) the different survival rates of faecal coliforms and faecal streptococci. Whereas in human faeces, faecal coliforms outnumber faecal streptococci, of which the enterococci predominate (Cooper and Ramadan, 1955; Kenner et al., 1960; Mead, 1965; Noble, 1978), in the faeces of animals, particularly cattle, faecal streptococci outnumber the faecal coliforms of which the non-enterococci predominate (Cooper and Ramadan, 1955; Geldreich and Kenner, 1969). The simultaneous enumeration of faecal coliforms and faecal streptococci in polluted water enables the FC to FS ratio to be calculated, and this ratio affords a means of estimating whether the pollution originated from a human or non-human source. Kjellander (1960), Raibaud et al. (1961) and Geldreich (1966) have shown that a FC:FS ratio of less than 0.7 usually indicates contamination from domesticated animals, whereas a ratio of greater than 4 indicates a human source (Geldreich and Kenner, 1969). Thus, it is theoretically possible to ascribe pollution to human or to an animal source on the evidence of the FC:FS ratio provided the pollution is known to be of recent origin. Geldreich and Kenner (1969) recommended that the initial FC:FS ratio should only be considered if samples are taken in the first 24h following the discharge of bacteria into the water course. The obvious drawback is the reported survival rates of faecal coliforms and faecal streptococci in natural waters. The enterococci survive better than faecal coliforms which in turn survive better than the non-

enterococci (Kjellander, 1960; Geldreich and Kenner, 1969; McFeters et al. 1974). Also, it is not always possible to judge the age of the pollution and estimate the time between excretion and discharge into the water course.

1.8. The Value of Bacterial Classification

The structure of a bacterium offers few distinguishing characters, therefore, bacteriologists rely on physiological, antigenic, biochemical and other characters to differentiate one group of bacteria from the other. Employing a combination of these methods, bacteriologists have been able to differentiate a large number of bacterial species and strains. The criteria that have determined the classification and nomenclature of bacteria are not, therefore, such as would be accepted by the systematist in other branches of biology. Numerous attempts have been made to introduce order into the classification of bacteria based on those of the zoologist and the botanist. But bacteria pose greater problems than higher organisms because the study of their evolution is exceedingly difficult; a phylogenetic basis for classification is impossible.

1.8.1. Classification

Classification is the orderly arrangement of as many easily-determined characters as possible so that bacteria can be arranged into groups of organisms that share common properties. Bacteria are conveniently divided into two large groups based on their reaction to Gram's method of staining. Most genera are made up of bacteria that are either gram-positive or gram-negative, so that organisms can be distributed according to the gram-reaction of the majority species they contain, whereby adjacent genera show some similarities but the more distant ones have less in common. Classification, therefore, is the process of

recognising and describing groups of living organisms and is a part of the wider study of systematics as shown below.

Systematics
- i) Classification
- ii) Taxonomy
- (iii) Identification
- (iv) Nomenclature
- (v) Phylogeny (Sneath and Sokel, 1973.)

The establishment of principles and rules of a workable classification and the methods of naming the groups so classified is in the province of taxonomy. But what is taxonomy? Cowan (1974) in his definition of taxonomy likened taxonomy to a cocktail - a mixture of three skillfully blended components.

i) Classification, or orderly arrangement of units; ii) nomenclature, or naming of the units and iii) identification of the unknown with a unit defined by (i) and (ii). To Cowan's definition may be added, assembly of a collection representing as adequately as possible all units previously named and described. Then his first ingredient would be the second and his second the third and his third the fourth. Taxonomy, therefore, provides the microbiologists with the names of the micro-organisms with which they are working, and gives meaning to those names by furnishing descriptions of the micro-organisms to which the names are assigned. So the taxonomist prepares a dictionary, or a portion of a dictionary of names and definitions. If the definitions are poor the names are meaningless.

But what are the units Cowan refers to in his definition? Cowan accepted species as a convenient unit. And how is species defined? The International Code of Nomenclature of Bacteria and Viruses (1948, 1958), defined a species as dependent on the opinion of the investigator. But a species should be defined as a group of strains, a population of freshly isolated strains, old stock strains and their variants, that have in common a set of correlating characteristics that separate them from other groups of strains. Consequently, to describe such a species, one must study newly isolated strains, old stock strains and as many of their variants as possible and then select correlating properties that are common to these strains for delineating the species these strains represent. As a result,as long as a strain is in existence, it is recognisable as belonging to the same species and bears the species name. Its species name does not change because the strain has lost some variable property such as haemolytic activity or even pathogenicity.

Again to quote Cowan,"There is not one classification (made by God, nature or by man) but any number of classifications, all made by men, each with a particular purpose in mind." Just as there are classifications of Kingdoms, there are classifications of genera and classifications of specific groups of bacteria, such as the 'coliforms' and the 'faecal streptococci'. And each particular classification has a purpose in mind. For example, the streptococci have been subjected to several

types of investigations in order to classify them. These include numerical taxonomic studies Colman (1968), G + C% base ratio studies; nucleic acid homology studies (Roop et al. 1974) and studies of biochemical pathways and electron transport systems.

A large number of micro-organisms will not fit into neat, separate, labelled compartments since "the different kinds of bacteria are not separated by sharp divisions but by slight and subtle differences in character so that they seem to blend into each other and resemble a spectrum" (Cowan, 1974). Therefore, the greatest need for classification is the use of as many well-determined characters as possible. These characters may be specific, distinguishing, or characters shared by all members of a group. When this is done, then the identification process becomes meaningful.

1. 9. The Need for Pure Cultures in Identification

Microbiologists encounter a great deal of difficulty in identification due to a mixed culture as a starting material. Before initiating any detailed examination of an unknown bacterial isolate the purity of the culture must be confirmed; this is of particular importance if colonies have been picked from a selective medium. Colonies picked from a selective medium are replated on a non-inhibitory medium or preferably on an indicator medium. Usually, if all the colonies have a uniform appearance the culture can be assumed to be pure. A pure culture retains its original characters because the chances are, on an ordinary nutrient medium, millions to one against picking the mutant (Cowan, 1974). The unknown bacterium in pure culture is then subjected to the tests used for the identification.

1. 10. System of Identification of Bacteria

Several different criteria have been used by bacteriologists to identify an unknown bacterium. These criteria include:

(i) morphology and staining reactions

(ii) cultural characteristics

(iii) biochemical reactions

(iv) antigenic characters

(v) immunofluorescence procedures

(vi) typing methods

(vii) animal pathogenicity

(viii) antibiotic sensitivity

The first four criteria were employed in the identification procedures during this study. The biochemical tests used in the identification of isolates are given in the "Materials and Methods Section". Composite reviews of such tests have been published by Blazevic and Ederer, (1975).

1. 11. The Emergence of Antimicrobial Agents

Long after Ehrlich's pioneering work resulted in the discovery of arsphenamine, a cure for syphilis and other spirochaetal diseases, a paper appeared in 1935 introducing Prontosil, the first of the sulphonamide drugs. Since then, a large number of sulphonamide derivatives had become available and they had been used effectively to reduce the high mortality rate of various bacterial infections. At the end of that period the antibiotic penicillin came on to

the scene as an antibacterial agent. Since then, there has been a rapid discovery and synthesis of a variety of antimicrobial agents and more recently, by the chemical modification of the existing antimicrobial agents many more are being synthesized. The development of penicillin and other antimicrobial agents to control diseases in man was quickly extended to provide similar benefits for animals. It is a requirement of cardinal importance that a drug must exhibit a high degree of selective toxicity.

1.12. Mechanisms of Action of Antibacterial Agents

An antimicrobial agent selectively interferes with one or more of the processes essential for the life of a microorganism without having any significant effect on the corresponding processes within the host. As a result the micro-organism is killed-bactericidal or becomes incapable of dividing-bacteristatic, and is eliminated by the defences of the host. Practically all of the important metabolic processes in bacterial cells are accessible to some antimicrobial agent. The site of action of most of the important drugs is now well established. However, the targets attacked relate to one or more of the following metabolic activities taking place within the bacterial cell:

1. DNA synthesis
2. Protein synthesis
3. Biochemical transformations
4. Synthesis and function of the cell membrane
5. Growth and integrity of the cell wall
6. RNA synthesis.

All these processes are mediated by enzymes and for the great majority of antibiotics, the point of attack is at the surface of one or more of the vital metabolic enzymes. It is customary to group antibacterial agents according to the principal processes they interfere with. But

within this grouping individual antibacterial agents differ in their precise mode of action.

A concise version of the mode of action of antibacterial agents is presented. Special regard is given to the antibacterial agents employed in the course of this investigation.

1.12.1. Inhibitors of DNA Synthesis

Nalidixic acid is an effective and specific inhibitor of DNA synthesis in bacteria (Gross et al.,1965). It is believed that it exerts its antibacterial action by binding to DNA polymerase thereby preventing the growth of DNA strands. Because DNA replication does not take place, the bacterium is unable to reproduce itself and ultimately dies. The exact mode of action is still unknown, but the action of nalidixic acid on DNA has proved to be of use in studies of bacterial conjugation (Barbour, 1967). Although the basic mechanism of DNA replication in human and bacterial cells is very similar, nalidixic acid does not bind to human DNA polymerase and therefore, has no significant effect on the human host.

5-iodo-deoxyuridine is known to interfere with viral DNA. Phleomycin, novobiocin and hydroxyurea are also known to inhibit bacterial DNA synthesis.

1.12.2. Inhibitors of Protein Synthesis

(a) Erythromycin Erythromcycin prevents the translocation event (Gale et al.,1972) in protein synthesis from taking place by binding to the 50S ribosomal subunit. Erythromycin acts selectively against bacteria for two reasons: (i) it binds only to the ribosomes of the organism and not to the cytoplasmic ribosomes of the host cell which differ from them in chemical structure; (ii) although human mitochondrial ribosomes closely resemble their bacterial counterparts, erythromycin is incapable of penetrating the mitochondrial membrane and is therefore incapable of reaching them.

(b) Streptomycin The aminoglycoside, streptomycin inhibits protein synthesis and decreases the fidelity of translation of the messenger RNA (Carner and Kogut, 1980).

It prevents amino acid polymerisation after formation of the initiation complex by binding to the S12 protein of the 30S ribosomal subunit. Mutations in the gene coding for this specific ribosomal protein control the binding of this drug to the ribosome and hence the sensitivity of the drug. Misreading of the genetic code to produce "nonsense" proteins was thought to be the lethal action of this drug, but this appears not to be the case. Its bactericidal action, though still unexplained, may be due to the lack of production of required proteins. High level of resistance to streptomycin is by a single step mutation in the bacterial genome coding for S12. The ribosomes are unable to bind to streptomycin because the structure of the S12 protein is altered. Alternative mechanisms of resistance to streptomycin are described later. Other aminoglycosides, for example gentamicin, also act directly on the ribosome where they inhibit protein synthesis usually by a mechanism similar to that of streptomycin.

(c) Tetracycline Tetracycline also binds to the 30S subunit of the ribosome and blocks the uptake of aminoacyl t-RNA to the A site. The precise mechanism responsible for this action is still obscure but it is known to differ from the mechanism by which streptomycin achieves a similar end.

1.12.3. Antibacterial Agents Affecting Biochemical Transformations

(a) Sulphonamides The sulphonamides prevent the normal utilization of para-aminobenzoic acid (PABA) by bacteria. Sulphonamides act by competitively inhibiting dihydropteroate formation. The structure of sulphonamides closely resemble that of PABA which is a precursor of folic acid. In the metabolic pathway for the synthesis of folic acid, the pathways of pteridine and PABA merge. The enzyme at the junction of the

two pathways has two receptor sites - one for pteridine and the other for PABA. When the two substrates are in their respective sites, they combine to form tetrahydropteroic acid. This molecule is then freed from the enzyme and moves on to the next enzyme in the pathway where it combines with glutamic acid to form pteroylglutamic acid, that is, folic acid. Folic acid in its turn participates in a number of other metabolic transformations, amongst them the biosynthesis of thymine which is one of the bases essential for DNA synthesis.

Figure 1.1.

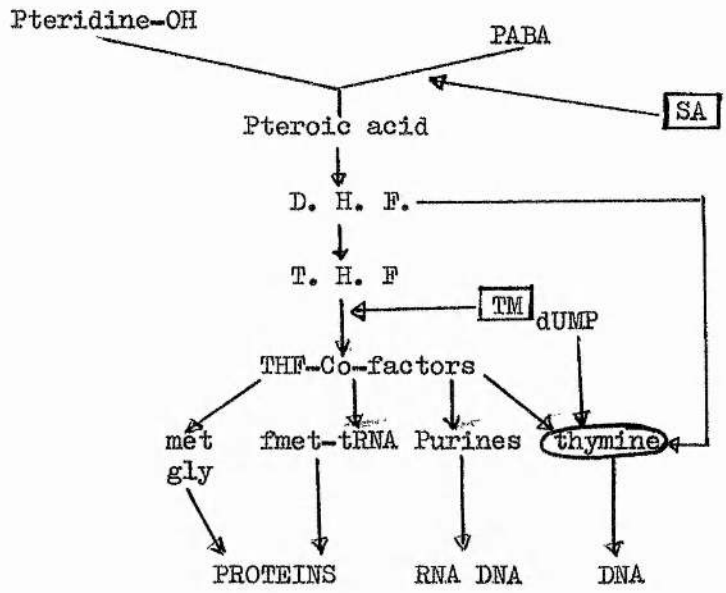


Figure 1.1. Some of the most important reactions that are dependent on tetrahydrofolate cofactors and inhibited by sulphonamide (SA) or trimethoprim (TM). After Then and Angehrn, 1973.

When a sufficiently high concentration of a sulphonamide is introduced into a bacterial cell, because of their structural similarity to PABA the sulphonamide binds to the receptor on the enzyme thus preventing the formation of tetrahydropteroic acid by competitive inhibition. As a result further synthesis of folic acid cannot take place. In the absence of folic acid, thymine synthesis is halted and DNA replication is disrupted.

(b) Trimethoprim Trimethoprim inhibits the enzyme dihydrofolate reductase in the bacterial cell. This enzyme is required to catalyse the reduction of dihydrofolate to tetrahydrofolate and is essential in the biosynthesis of purines, pyrimidines and certain amino acids (Fig. 1.1). Since both sulphonamide and trimethoprim act on different enzymes within the folic acid biosynthetic pathway, their combined action is synergistic and for this reason, preparations such as co-trimoxazole have been marketed for therapeutic use.

1.12.4. Antibacterial Agents Affecting Cell Wall Synthesis

(a) Penicillins The penicillins are believed to weaken the cell wall by binding to and inactivating the cross-linking enzyme transpeptidase in actively growing and dividing cells. This prevents the glycine-alanine bonding reaction from taking place. In the growing cell lytic enzymes continue to open up the peptidoglycan network and new subunits continue to be incorporated, but in the presence of penicillin these cannot be cross-linked to form the rigid network. As a result, the cell wall is progressively weakened. With further growth the cell wall is no longer able to withstand the high internal pressure and the wall ruptures leading to cellular lysis and cell death. Ampicillin is a derivative of penicillin in which the side chain on the β-lactam ring has been chemically modified. Penicillins do not affect mammalian cells since the latter do not synthesise peptidoglycan nor do they possess a cell wall.

(b) Cephalosporins The Cephalosporins, for example cephalexin, possess a similar mode of action to the penicillins on bacterial cell wall synthesis. They are, however, often unsusceptible to the destructive effect of the bacterial enzyme penicillinase, but may be sensitive to cephalosporinases.

1.13. Evolution of Drug Resistance

Before the discovery of antibacterial agents, physicians had to know the infecting bacterium and its serological type so that the correct serum therapy could be prescribed. With increase in usage of chemotherapeutic

agents, the physician's prime concern was no longer the identity of the infecting bacterium and its antigenic type but was for the sensitivity and/or the resistance of the bacterium to the then available drugs. Shortly after the introduction into clinical practice of new antibacterial agents, with an alarming frequency, clinicians encountered micro-organisms that had become resistant to the "wonder drugs" of a few months ago and in some cases the drugs had become useless. Both intrinsic and acquired bacterial resistance to therapeutic agents was recognised many years before the introduction of the sulphonamides. Even in the early days of the introduction of penicillin into clinical practice certain bacteria were not killed by this agent (Abraham and Chain, 1940), and some seemed insensitive while yet others were capable of actively destroying the antibiotic presumably by enzyme action. The introduction of new antibacterial agents eclipsed this phenomenon and the first period of disillusionment came in the 1950's with the development of staphylococcal resistance to many agents. At present, the problem of bacterial resistance to some drugs has reached alarming proportions, and we are faced with multiple resistance. Multiple resistance is not only found in Staphylococcus aureus but also in gram-negative rods and in other unrelated genera with the exhibition of resistance to the commonly used drugs both in hospitals and in the community.

The emergence and spread of resistant bacteria have been shown to be related to drug usage. An overview for gram-negative rods is given by Richmond (1972); Balows (1977); and Ayliffe et al. (1977) provide that for staphylococci. Acquisition of drug resistance by conjugation was first reported by Japanese workers who demonstrated multiple drug resistance transfer from E. coli to Shigella (Watanabe, 1963). The introduction of a new agent may be followed rapidly by resistance or

there may be a short "honeymoon" period during which the drug is effective or it may be effective for many years. After this "honeymoon" period, an extremely high proportion of totally resistant bacteria becomes evident and the value of the antibacterial agent as a chemotherapeutic agent is severely undermined. Resistance of Staphylococcus aureus to penicillin provides an obvious example. Soon after the introduction of benzyl penicillin into widespread use, a large scale World Health Organization survey showed 8% of staphylococcal strains to be resistant (Munch-Petersen and Boundy, 1962). The present level is about 70% and a similar pattern is found with other antibacterial agents. Resistance to gentamicin is another example. After 10 years of use of this antibiotic, with almost no resistance, resistance has been seen in Pseudomonas aeruginosa, Staphylococcus aureus and in Klebsiella aerogenes. The term resistance as used in this context refers to those species and their variants that are capable of growth in the presence of a given antimicrobial agent at a concentration significantly higher than a known sensitive strain of the same species. Decline in resistance may occur inexplicably without changes in antibiotic use, but frequently decline follows restriction of use of the antibiotic.

1.14. Mechanisms of Resistance

Since the great majority of antibiotics act by attacking protein targets, the resistant bacterial cell has to inhibit such action. There are a variety of possible mechanisms by which this is achieved:

(i) alteration of the target enzyme/protein to preclude drug binding

(ii) provision of an alternative metabolic pathway

(iii) restriction of access to the target

(iv) inactivation of the antibiotic by enzymes.

The first evidence in medical practice of the ability of micro-

organisms to become resistant against man-made antibacterials was resistance to sulphonamide in the Pneumococcus. This is due to an alteration in the target enzyme for sulphonamide attack. The development of bacterial strains resistant to sulphonamides is believed to be due to mutations occurring in the DNA segment responsible for the synthesis of the enzyme blocked by sulphonamides. This leads to the synthesis of an equivalent enzyme which remains capable of reacting with para-aminobenzoic acid but to which sulphonamide can no longer bind. In these circumstances, folic acid biosynthesis continues. Although relatively uncommon, resistance to trimethoprim, on the other hand, is achieved by the provision of an alternative version of the target enzyme which makes the drug resistant (Garrod et al.,1973). The concomitant administration of trimethoprim with a sulphonamide produces a second block at another point in the pathway for folic acid biosynthesis.

Another instance of a high level of resistance being displayed as a result of an alteration in the drug target is that of high resistance to streptomycin as a result of a single point mutation producing an altered S12 protein of the 30S ribosomal subunit to which the drug cannot bind.

Tetracycline resistance is achieved by preventing the antibiotic from reaching an inhibiting concentration in the cell i.e. prevention of access to the target. One major problem would seem to be the identification of the membrane proteins, the production of which is coded by R-factors providing tetracycline impermeability of the cell membrane and consequently the cell resistance (Navashin et al.,1975).

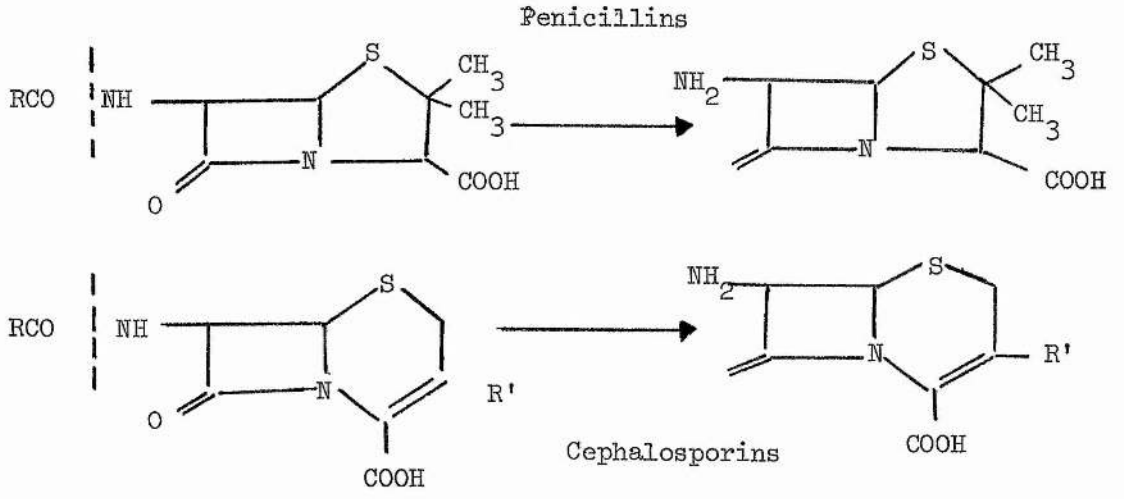
The lipopolysaccharide layer of the gram-negative bacterial cell wall has often been referred to as a permeability barrier to the uptake of certain drugs to explain the apparent lower sensitivity of many gram-negative species to antibiotics as compared with gram-positive species.

The activities of the penicillins, cephalosporins and amino-glycosides may be inhibited by their conversion to inactive metabolites by enzyme action in resistant micro-organisms. Such drug-inactivating enzymes may act in two main ways: either they substitute key residues on the antibiotic molecule, or they inactivate the antibiotic by opening one or more covalent bonds in the structure.

The former group are best illustrated by the aminoglycoside inactivating enzymes which include acetyltransferases, phospho-transferases and adenylylating enzymes. Not all of these enzymes are equally effective against all of the different aminoglycosides. Thus resistance to one member does not necessarily imply that resistance will be shown to all members of the group. It is worth noting that with aminoglycosides, for example streptomycin, various levels of resistance may be seen depending upon the mechanism of resistance involved.

The latter group involve enzymes which split covalent bonds and are best illustrated by a consideration of the β-lactam antibiotics, penicillins and cephalosporins. Such enzymes include acylases, esterases and β-lactamases.

Acylases hydrolyse the 6-acyl group of penicillins or the 7-acyl group of cephalosporins.

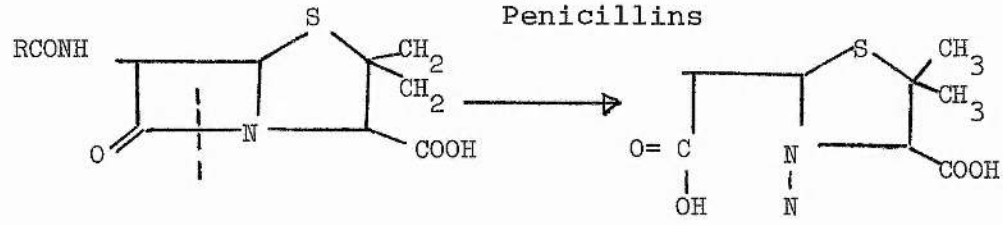


They are produced by bacteria and fungi and are side-chain specific.

Removal of the acyl side chain often results in loss of antibacterial acitivity.

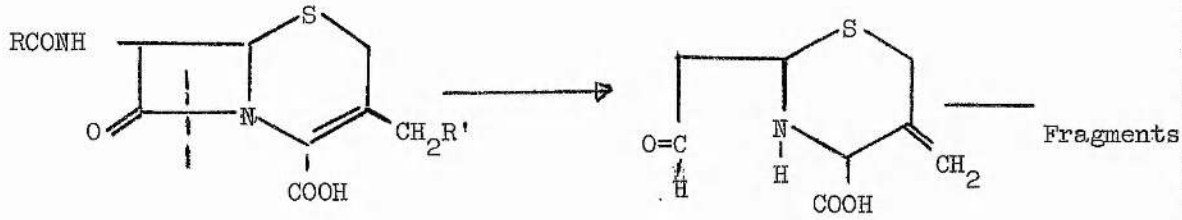
β-lactamases hydrolyse the amide bond in the β-lactam ring of penicillins and cephalosporins.

Penicillins



Penicilloic acid

Cephalosporins



There may be three types of β-lactamases, penicillinases, cephalosporinases and a third relatively non-specific enzyme. Both gram-positive and gram-negative micro-organisms show susceptibility to β-lactamases. Among the β-lactamase-producing gram-positive organisms, Staphylococcus aureus is the only major pathogen. These organisms produce inducible extracellular β-lactamases of which there are four major variants. Among the gram-negative organisms the situation is more complex. The enzyme can be divided into 5 major classes (Matthew, 1979) on the basis of a number of arbitrary parameters, for example, substrate profile and response to inhibitors.

1.15. Determination of Resistance

A number of considerations are involved in selecting an appropriate antimicrobial agent to treat an infection. These are:

(i) knowledge of the inherent susceptibility of the infecting organism to appropriate antimicrobial agents

(ii) clinical pharmacological properties, for example, toxicity, protein binding, distribution, absorption and excretion

(iii) previous clinical experience of efficacy in treating infections due to the same species

(iv) the nature of the underlying pathological process, its natural history and its influence on chemotherapy

(v) the immune status of the host. (Sherris, 1974).

Of these factors, the amount of antimicrobial agent required to inhibit or kill the organism in vitro and the level of antibiotic attained in the body fluid of the host during treatment are subject to measurement in the laboratory. Of the two factors, the former is directly concerned in this investigation and the procedure for this purpose is presented.

Different procedures have been developed for the determination of the susceptibility of organisms to antibacterial agents. However, the results of such measurements are not absolute values, because they are sometimes influenced markedly by the test conditions used. Differences in such factors as inoculum size, medium constitution, pH, atmosphere, incubation time and stability of antibiotic may all affect the amount of antibiotic required to inhibit the organism in vitro. Thus, the minimum inhibitory concentration (M.I.C.) of an antibiotic for an organism has to be defined according to the conditions of the test (Ericsson and Sherris, 1971).

Three basic methods, broth dilution, agar dilution and agar diffusion are in general use although they are by no means the only methods available. The procedures described for the dilution tests are derived from those recommended in the report in an International

Collaborative Study (Ericsson and Sherris, 1971). These procedures have gained considerable acceptance, and when performed as directed, and controlled with recommended standard strains give good reproducible results. The agar dilution procedure was adopted during this investigation.

1.15.1 Dilution Tests

Dilution tests are used to determine the minimal concentration of an antimicrobial agent required to inhibit or kill a micro-organism. The "agar dilution" method is useful in that a number of strains can be tested simultaneously. Appropriate dilutions of the antibiotic are prepared at 10 times the concentration required in the final test. The minimum inhibitory concentration is the lowest concentration of the antibacterial that completely inhibits or allows a given number of organisms to grow (see Results Section). The dilution test can be performed in liquid (serial dilutions), or agar medium. When performed in agar medium it is referred to as "agar dilution" method. Both terms are actually misnomer because it is the antibacterial agent that is being diluted rather than the agar or broth.

1.15.2. Diffusion Tests

The diffusion test procedure is accepted by the Food and Drug Administration (1972; 1973), in the United States of America and proposed as a tentative standard by the National Committee on Clinical Laboratory Standards' Subcommittee on Antibiotic Susceptibility Testing (1973). In this method the antibiotic diffuses from a focus through a solid medium, inhibiting the growth of an organism. When filter-paper disc containing fixed amount of antibiotic are applied to the agar surface inoculated with the test organism, the antibiotic diffuses into the surrounding medium, at the same time the micro-organisms are

multiplying logarithmically on the agar surface. Growth will be inhibited in the area where sufficiently high concentration of drugs have been obtained, and where the concentration of drug is not great enough there will be growth. The major problem associated with this method is in attaining absolute reproducibility of agar thickness in the plates when one considers that the recommended agar thickness is 4mm.

At present there appears to be no consensus on the conditions for susceptibility testing for obligate anaerobic organisms although much work is in progress in the United Kingdom and in the United States of America.

1.16. Bacterial Plasmids

Resistance plasmids are fundamental to any understanding of the problem of bacterial drug resistance, particularly multiple drug resistance. Since resistance plasmids belong to one of a number of classes of bacterial plasmids, information concerning plasmids in general is likely to contribute significantly towards our understanding of resistance plasmids.

Bacterial plasmids are autonomous DNA elements which replicate independently of the bacterial chromosome (Novick et al., 1976). They are sometimes called supernumerary chromosomes, but are smaller than the bacterial chromosome. In general plasmid size seems to show a continuous spectrum from a MW of less than a million to 100 million or more. Two basic types of plasmids are represented from their sizes.
(i) The small plasmids (MW 1×10^6 to 10×10^6) which are present in the bacteria as multiple copies (Cozzarelli et al., 1968; Clewell and Helinski, 1970; Milliken and Clowes, 1973; Guerry et al., 1974; Kool and Nijkamp, 1974; Smith et al., 1974).
(ii) The larger plasmids (10×10^6 and upwards) which are usually

only present as one or at the most a few copies per cell (Hickson et al.,1967; Freifelder and Freifelder, 1968; Nisioka et al.,1970; Vapnek et al.,1971; Grinsted et al.,1972). These two types have distinct modes of replication. Replication of the second type is essentially the same as that for chromosome replication. The manner of replication of the first type differs in several respects from replication of the chromosome. These differences as a consequence may gave different evolutionary implications (Bennett and Richmond, 1979).

Bacteria plasmids may be distinguished according to the genetic determinants they carry, for example:

(i) genes specifying drug resistance
(ii) genes specifying bacteriocin production
(iii) genes specifying metabolic enzyme synthesis
(iv) genes specifying pathogenicity
(v) genes specifying heavy metal resistance
(vi) cryptic plasmids

However, within these broad sub-divisions are to be found many different types of plasmids. One of the more useful criteria available at present which can be used to classify bacterial plasmids is the phenomenon of compatibility. Certain pairs of plasmids are unable to coexist stably in some bacterial strains, while other pairs persist for long periods and do not influence adversely the survival of one another at all (Novick, 1969; Clowes, 1972; Helinski, 1973; Falkow, 1975).

The compatibility test, separates plasmids into large numbers of groups. If two plasmids can be maintained in the same bacterium, in the absence of any selection for either plasmid, then they are said to be compatible and so belong to different compatibility groups. On the other hand, if two plasmids cannot be maintained in a bacterium, except with continued selection for both, then they are said to be incompatible and so belong to the same compatible group. (Novick and

Richmond, 1965; Datta, 1974; 1975). At present more than one plasmid compatibility group is known to exist among the various members of the Enterobacteriaceae notably *E. coli* K12 (Datta, 1974), among strains of *Pseudomonas aeruginosa* (Chakrabarty, 1976), in *Streptomyces coelicolor* (Schrempf *et al.*,1975; Kirby, 1976), and among strains of *Staphylococcus aureus* (Novick and Richmond, 1965; Novick and Brodsky, 1972). There is evidence that incompatible plasmids do share homologous DNA sequences (Guerry and Falkow, 1971; Grindley *et al.*, 1973; Crosa *et al.*, 1973; Falkow et al., 1974). The objectives of such classification are two-fold: (i) is to establish whether resistance in a given outbreak of infection can be traced back to a single source and (ii) to establish the manner and rate of plasmid evolution (Broda, 1979).

1.17. Transfer of Resistance Genes

The most important feature of plasmid behaviour is probably their ability to be transferred between bacterial cells. Under certain circumstances transfer between members of widely different bacterial genera may occur (Grinsted *et al.*, 1972; Olsen and Shipley, 1973). The host range of some plasmids e.g. RP4, is so wide that it seems hard to find a species of gram-negative bacteria that is not accessible to them (Olsen and Shipley, 1973). Many bacterial plasmids carry information which allows them to direct the production of a specific mechanism whereby the plasmid can be transferred from one bacterial cell to another (Watanabe, 1963; Novick, 1969; Clowes, 1972; Helinski, 1973; Achtman and Helmuth, 1974). Such plasmids are said to be self-transmissible and transfer of a plasmid in this way is known as conjugal transfer or infective transfer and is a highly sophisticated means of exchanging genetic information. Self-transmission via conjugation is thought to be largely responsible for the widespread dissemination of

drug resistance throughout the bacterial kingdom.

There are three broad types of genetic material transfer between bacteria that have been reasonably well characterized.

1.17.1. Transformation

Griffith (1928) described the permanent transformation of the capsular material of a living Pneumococcus by materials derived from dead Pneumococcus of another type. Avery et al. (1944) made a further advance when they identified the transforming material as DNA. Transformation experiments carried out by Hotchkiss and Gabor (1970) provided the formal proof that DNA was the source of inherited information in bacterial cells. In transformation naked plasmid DNA passes between donor and recipient and differs from both conjugation and transduction (see below) in that it does not appear to be restricted to transfer between cells of closely-related species, and it has recently become of great importance in the laboratory analysis of functions of individual DNA molecules.

1.17.2. Transduction

In transduction, the plasmid DNA passes from donor to recipient by means of phage infection (Zinder and Lederberg, 1952; Hayes, 1968). A phage attaches to a cell and some of that cell's genes are packaged in daughter phage particles and can then be transferred by the phage to a second cell. This method of transfer is important especially for transfer of plasmids between Staphylococci, where until recently, no conjugal transfer was thought possible (Naidoo and Noble, 1980). Unlike transformation, there is every reason to believe that effective gene transfer by transduction does occur in nature (Hayes, 1968).

1.17.3. Conjugation

Conjugation has been known for many years in the Enterobacteriaceae, Pseudomonas, Neisseria and recently in Staphylococci and Streptococci. Although conjugation is basically similar in its overall consequences

in terms of drug resistance transfer, to transformation and transduction, the process shows several differences with respect to the mechanisms involved.

Conjugation requires physical cell-to-cell contact (Davis, 1950) which in effect leads to specific pair formation. Specific pairs do not necessarily conjugate. The effective pair formation stage requires the formation of a specific conjugation tube which ensures that genetic material can pass from donor cell to the recipient cell. The DNA may enter via the pili channel or by direct wall-to-wall contact. At the resistance transfer factor mobilisation step an enzymatic nick of the super coiled plasmid DNA produces an open circular form and exposes a 5'-phosphate end to enter the recipient first. The entry of the single stranded DNA is accomplished by the synthesis of a complementary strand and the formation of a covalently closed circular molecule.

There are many barriers to successful transfer between bacteria. These include surface exclusion and some cases where the strains may be unable to make contact between unrelated strains. Once inside the recipient cell, DNA may be subject to restriction by enzymes. Also it must either be able to replicate as a plasmid or integrate into the host chromosome. If it must be able to replicate itself it must be able to use the host machinery or provide its own. It must also be compatible with any plasmids that already are present or displace them.

This study was initiated to:

(i) determine the species distribution of coliforms and group D streptococci isolated from water samples taken from above and below a sewer outfall;

(ii) determine their susceptibilities to several antibacterial agents;

(iii) determine to what extent effluents from domestic wastes may serve as reservoir for the spread of antibiotic resistant coliforms and group D streptococci;

(iv) determine the transferability of drug resistance factors in both groups of organisms, and the isolation of plasmids from drug-resistant group D streptococci.

Finally, as a pilot study, the survival of (a) coliforms in water under natural conditions and (b) the survival of non-sporing anaerobic bacteria during sewage treatment process was also investigated.

The difficulties associated with such study have their origins in the failure of earlier workers to adequately characterise the composite members of the coliforms and group D streptococci associated with sewage-polluted waters. These difficulties also apply to the susceptibility of both groups of organisms to antibiotics. Hence it is difficult to find reports in the literature pertaining to such study. Although information regarding the susceptibility of both groups of organisms to antibiotics is available, such information generally applies to clinical isolates. Therefore a comparative study of (a) species distribution and (b) antibiotic susceptibility of presumptive group D streptococci isolated from a variety of clinical sources was also undertaken.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Sterilization of Materials

Amongst the usual methods of sterilization those employed during this investigation included autoclaving, steaming, boiling, dry heat and filtration. Media, unless otherwise stated, and the Millipore filtration equipment, were sterilized by autoclaving at 121°C for 15 min (15 p.s.i. steam pressure). Glassware was sterilized in a dry heat oven at 160°C for 2h.

The methods of sterilization used for carbohydrate solutions were dependent upon the properties of the individual sugars and these are described later in the appropriate section of the work.

2.1.1. Microscopic Analysis

A Gillett and Sibert microscope was used for all gram stain and hanging drop observations. A zoom stereo binocular microscope (Kyowa Optical Ltd.) or a Gallenkamp colony counter was used for colony counting.

2.1.2. Chemicals

Wherever possible analytical grade chemicals were used.

2.1.3. pH Measurements

These were performed on a PYE pH meter.

2.1.4. Absorbance Measurements

Unicam SP600 Series 2 spectrophotometer was used for absorbance measurements.

2.2. Reagents

For the characterization tests of the coliform isolates the reagents used, their formulae and method of preparation are given below. The formulae were obtained from (Cowan, 1974).

BENEDICT'S QUALITATIVE SOLUTION

Sodium citrate	17.30g
Na_2CO_3 anhydrous	10.00g
$CuSO_4.5H_2O$	1.73g
Distilled water	100 ml

The sodium citrate and carbonate were dissolved in 60ml distilled water. The copper sulphate was dissolved in 20ml water and added with constant stirring to the first solution. This was made up to 100 ml with water and stored at room temperature.

EHRLICH'S REAGENT

p-dimethylaminobenzaldehyde	1g
Absolute ethanol	95ml
Concentrated hydrochloric acid	20ml

The aldehyde was dissolved in the ethanol and the acid added. The reagent was stored wrapped in aluminium foil at room temperature.

METHYL RED SOLUTION

Methyl red	0.04g
Ethanol	40ml
Distilled water	60ml

The methyl red was dissolved in the ethanol and the distilled water added and was thoroughly mixed.

α-NAPHTHOL SOLUTION

α-naphthol	5g
Ethanol	100ml

5g of the α-naphthol was dissolved in 100ml ethanol mixed and stored in a dark container at room temperature.

POTASSIUM HYDROXIDE SOLUTION

KOH	40g
Distilled water	100ml

The KOH was dissolved in the water mixed and stored at room temperature.

OXIDASE TEST REAGENT

NNN'N'-tetramethyl-p-phenylenediamine dihydrochloride	1g
Distilled water	100ml

The reagent was dissolved in water, mixed, quickly dispensed into bijou bottles and stored frozen at -20°C. The reagent was always colourless when thawed for use.

HYDROGEN PEROXIDE SOLUTION

Hydrogen peroxide 100 vol	3 ml
Distilled water	97 ml

The peroxide was diluted with the water mixed and stored at 4°C in a dark container wrapped with aluminium foil.

LUGOL'S IODINE STOCK SOLUTION

Iodine	5g
Potassium Iodide	10g
Distilled water	100ml

The potassium iodide and iodine were dissolved in 10ml of water and made up to 100ml.

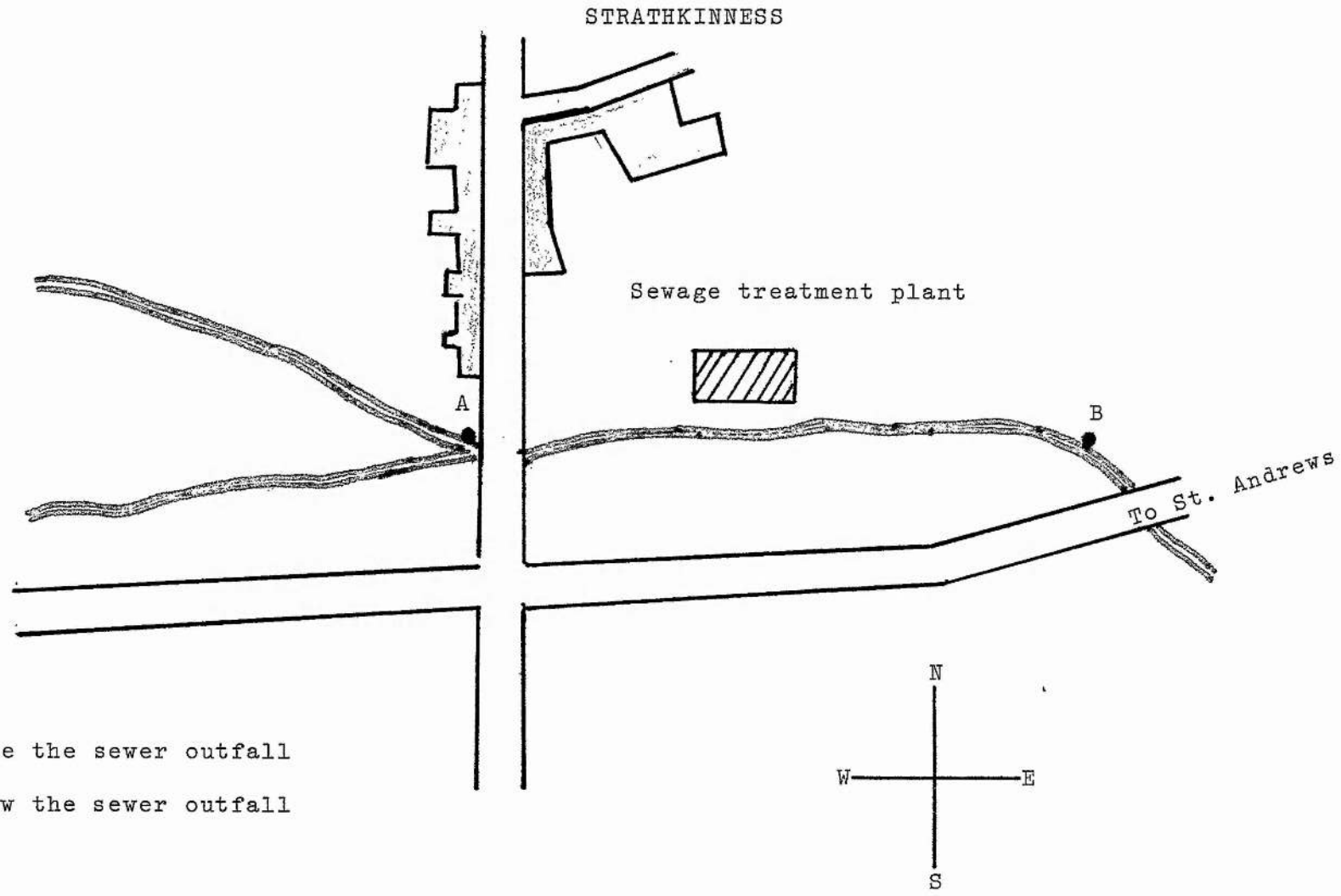
WORKING SOLUTION

10ml of the stock solution was diluted with 40ml of water before use.

2.3. Buffer Solutions

The buffer solutions used in this study were as given below. The solutions were prepared as recommended by Meynell and Meynell (1975) or by Cruickshank et al. (1975) to achieve the desired pH's. Citrate-phosphate buffers were used in the preparation of antibiotic stock solutions requiring a pH of 4.5 or pH 5.0., whereas phosphate buffers were used for the preparation of antibiotic stock solutions requiring a pH of 7.0 or 8.0. Tris-HCl buffer was used in the isolation procedure for plasmids. The stock solutions for these buffers were:-

FIGURE 2.1 LOCATION OF SAMPLING SITES ALONG THE KINNESSBURN



A. Above the sewer outfall

B. Below the sewer outfall

Buffer	Solution A	Solution B
Citric acid-phosphate	0.2M $Na_2HPO_4.2H_2O$	0.1M $C_6H_8O_7.H_2O$
Phosphate	0.2M $Na_2HPO_4.2H_2O$	0.2M $NaH_2PO_4.2H_2O$
Tris-HCl	0.2M HCl	0.2M Tris

Further details of their usage is given in the appropriate later sections.

2.4. Sampling Location

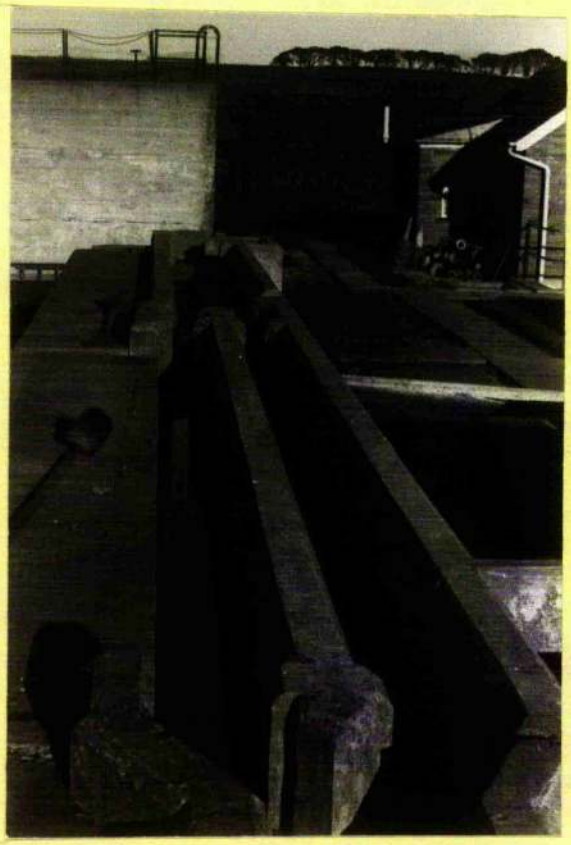
Figure 2.1 depicts the sampling locations in relation to the Strathkinness sewage works and St. Andrews. Sampling point A is at the southern end of Strathkinness village and approximately one quarter mile above the sewage treatment plant. The water here is mainly an admixture of natural source with agricultural drain-off from pastoral land, with overflows from a few septic tanks, either directly or indirectly into the burn upstream from this sampling point. Sampling point B was approximately one quarter mile downstream from the discharge pipe of the sewage plant into the watercourse, and was about one half of a mile downstream from the village sampling point. This point was far enough downstream to ensure complete mixing of the effluent and the water.

The lay-out of the sewage treatment plant is shown in Figure 2.2. Sewage treatment involved initial screening through a coarse grid followed by passage through two settling tanks in series. During this primary treatment, materials which can be settled are removed.

The liquor from the primary settling tanks then passes through two biological filter beds through rotating arms, which spray controlled amounts of sewage over beds of corase granite. The granite becomes coated with zoogleal film of microorganisms and protozoa that aerobically degrade the organic load in the liquor. Pathogenic bacteria are generally eliminated during this stage, but disease-producing viruses may survive. Pathogenic bacteria, which account

FIGURE 2.2 BASIC LAYOUT OF STRATHKINESS SEWAGE PLANT

RAW SEWAGE ——→



FIRST PRIMARY SETTLING TANKS IN PARALLEL

SECOND PRIMARY SETTLING TANKS IN PARALLEL

BIOLOGICAL FILTER BED

HUMUS SETTLING TANK

SEWAGE SLUDGE SOLIDS
ONTO DRYING BEDS

FINAL EFFLUENT INTO
THE KINNESSBURN

for only a small proportion of the total number of bacteria in faeces, are generally diluted by the water in the sewage. Most of them do not survive for long because they are poorly adapted for growth relative to the other microorganisms present. Pathogens are forced to compete for nutrients with the mass of bacteria present in the sewage which have adapted to grow best at the temperature and conditions prevailing. As a result, most of the pathogenic bacteria are grossly outnumbered and eliminated. Animal viruses, on the other hand, cannot replicate in the sewage, where they lack the appropriate hosts, but they survive for long periods and even chlorination does not eliminate them as they are secluded within small aggregates of materials which protect them. The combined filtrates from the biological filter beds then pass through a humus settling tank, and the final effluent discharged into the water course.

2.5. Sample Collection : Environmental

On eight separate occasions during the month of October through to November, 1979, representative samples of water from each sampling point were simultaneously collected into 500ml capacity bottles fitted with ground-glass stoppers. On two of these occasions sewage liquor samples were taken from the primary and humus settling tanks as well. Samples were always collected from the points between 10-11 a.m. and within 15 min of each other. The sampling bottles were kept unopened until the moment they were to be filled. Each bottle was held near its base with a rod and plunged neck downwards below the surface of the water, and turned until the neck pointed slightly upwards with the mouth directed to the current. Whenever samples were collected, an air space of at least 2 inches was left in the bottles to facilitate mixing of the samples by shaking prior to dilution. Samples were always processed within 2h of collection.

Clinical: One hundred isolates of presumptive group D streptococci from various clinical sources were obtained from the Victoria Hospital, Kirkcaldy.

2.5.1. Sample Preparation

Medical flats containing 90ml ¼ strength Ringers solution (Oxoid Ltd.) were prepared and sterilized at 121°C for 20 min. These were used in the preparation of serial decimal dilutions of the water samples before filtration. This solution is an isotonic diluent for bacterial cells and it prevents their rupture.

For filtration, grided Nuflow cellulose acetate membranes (Oxoid Ltd.) were placed between filter paper circles, wrapped in aluminium foil and sterilized by autoclaving at 110°C for 20 min. Where appropriate, Whatman grade 17 pads of 47mm diameter wrapped in aluminium foil and sterilized at 121°C for 20 min were also used.

2.5.2. Membrane Enriched Teepol Broth (METB) *

This was the selective medium used for the detection and subsequent enumeration and isolation of coliforms. A sterile 7.62% solution of METB (Oxoid Ltd.) containing 0.4% Teepol 610 (B.D.H. Ltd.) was prepared as directed by the manufacturer.

2.5.3. Slanetz and Bartley Medium (Membrane-Enterococcus Agar) *

This was the selective medium used for the detection and subsequent enumeration and isolation of group D streptococci. The Slanetz and Bartley medium (Oxoid Ltd.) was heated to dissolve the agar but was not autoclaved since such treatment reduces the efficiency of the medium by precipitating the tetrazolium chloride.

2.5.4. Sample Dilution and Filtration

Using a constant dilution factor and a fresh pipette at each transfer step, initially, serial decimal dilutions were prepared in triplicate for the purposes of filtration. Once parameters had been established, dilutions were prepared down to 10^{-4} and those between 10^{-1} and 10^{-4} inclusive were filtered.

* For details of composition see appendix B.

Filtration was performed through 0.45μm porosity Nuflow gridded membranes on a Millipore three-phase filter manifold. After filtration, of each sample, the funnel bowl was washed out with sterile water and after removal of the vacuum the membranes were aseptically transferred onto the relevant selective medium for incubation.

2.5.5. Incubation Period

For coliform counts, the petri dishes were incubated at 30°C for 4h and then transferred to 37°C for 14-20h. For the group D streptococci counts, the plates were incubated at 37°C for 4h and transferred to a 44-45°C incubator for 44h.

2.6. Counting of Colonies

A steroscopic microscope was used for colony counting on all selective media.

2.6.1. Coliforms

The Teepol in the medium was selective for microorganisms which are not coliforms and a change in colour of the indicator (phenol red) in the medium from red to yellow, indicated acid production due to lactose fermentation. Thus all yellow colonies on the membranes from suitable dilutions were counted.

2.6.2. Group D Streptococci

Whilst the azide in the medium is selective for microorganisms which are not D streptococci, the reduction of tetrazolium chloride in the medium to the insoluble formazan by the streptococci produced red or maroon colonies and such colonies were regarded as group D streptococci and were counted.

For both coliforms and group D streptococci membranes showing 20-200 colonies were used to calculate the mean count $100ml^{-1}$.* For statistical reasons the counting of such numbers of colonies gives a more reliable result (D.H.S.S.(Welsh Office), 1969).

* $100ml^{-1}$ is equivalent to per 100ml.

2.7. Isolation of Pure Cultures

From one of the sampling runs, pure cultures were obtained by picking off 100 individual colonies from membranes showing 20-200 colonies from each sampling site, and streaking these on MacConkey agar plates for the coliforms, and on blood agar plates for the group D streptococci. The plates were incubated at 37°C overnight and examined for their purity. A total of 200 viable microorganisms were recovered from each sampling site.

2.8. Preservation of Stock Cultures

Pure stock cultures were obtained by growing the coliform isolates on nutrient agar slopes and the group D streptococci isolates on blood agar plates. The coliform isolates were stored at room temperature in the dark whereas the group D streptococci were stored at 4°C.

2.9. Inoculum for Characterization Tests

For the subsequent characterization tests described below, unless otherwise stated, a well isolated colony from agar plates was picked off and inoculated into Brain Heart Infusion broth (Oxoid Ltd.). This was then incubated at 37°C overnight, and each test medium received a drop of the turbid growth from a pasteur pipette.

2.10. Coliform Characterisation Test Procedures 1

The tests outlined below were those deemed most suitable from those quoted by Cowan (1974). The expected results for the tests used in this investigation are shown in Appendix C (p.167).

2.10.1 Acid and Gas Production at 44°C

MacConkey broth purple (Oxoid Ltd.) was prepared, dispersed into bijou bottles with Durham tubes and autoclaved at 121°C for 20 min. The medium was inoculated and incubated at 44°C for 48h. Acid formation was indicated by a yellow colouration of the broth, and gas production was indicated by an amount of gas at least sufficient

to fill the concavity at the top of the Durham tube.

2.10.2. Indole Production at 44°C

Tryptone water was prepared according to the formula in (D.H.S.S.(Welsh Office), 1969). The broth was inoculated and incubated at 44°C. After 24h incubation 0.5ml of Ehrlich's reagent was added. A positive reaction (red colour in the surface layer) denoted the presence of indole. The constituents of Ehrlich's reagent are given in the reagent section so are the constituents of the other reagents used.

The above two procedures were used to identify E. coli strains amongst the isolates.

2.11. Coliform Characterization Test Procedures 2

All isolates not identified as strains of E. coli were then subjected to the following series of tests (procedures 2/3).

2.11.1. MR - VP Test

MR - VP medium (Oxoid Ltd.) was prepared, distributed into bijou bottles and autoclaved. The medium was inoculated and incubated at 37°C for 48h. The Methyl Red (MR) and Voges-Proskauer (VP) tests were performed separately. For the MR test, 3 drops of methyl red reagent were added to the bottle. A positive reaction was depicted by a bright red colour whilst a negative reaction gave a yellow or orange colour.

For the VP test (Barritt, 1936) 0.6ml of a 5%α-naphthol in absolute ethanol was added and well mixed by vigorous shaking. 0.2ml of a 40% KOH solution was added and the bottle was shaken vigorously again. A positive reaction was indicated by the development of a red colour within 5 min.

2.11.2. Citrate Utilization

Modified Koser's citrate medium was prepared as recommended in

Cowan (1974). The medium was inoculated using a saline suspension of the organism and a straight wire, and incubated at 37°C for 24 to 48h. Citrate utilization was indicated by growth and turbidity of the medium.

2.11.2. Indole Production at 37°C

The medium was the same as given previously. The test procedure was also the same with the exception of the incubation temperature which was at 37°C.

2.12. Coliform Characterization Test Procedures 3.

2.12.1. Gluconate Oxidation

Gluconate broth (Shaw and Clarke, 1955) was prepared as described in Cowan (1974), but sodium gluconate (B.D.H. Ltd.) was used instead of potassium gluconate. The broth was distributed in 5ml aliquots in test tubes and was inoculated with a loopful of colonies of the test organism removed from an agar plate and incubated overnight at 37°C. 1ml of Benedict's qualitative solution was added, mixed and the tube contents were boiled for 10 min. The formation of a brown-orange or tan precipitate indicated a positive result. With a negative result the solution remained blue.

2.12.2. Malonate Utilization

Malonate-phenylalanine broth (Shaw and Clarke, 1955) was prepared following instructions given by Cowan (1974) and distributed in 3ml aliquots into bijou bottles. The medium was inoculated and incubated at 37°C for 24h. A deep blue colour was recorded as a positive reaction and in a negative reaction there was no change in the medium which remained green.

2.12.3. Phenylalanine Deamination

To the negative malonate-phenylalanine broth, 0.2ml of 0.1N HCl was added followed by 0.2ml of a 10% $FeCl_3$ solution. The bottle was shaken and observed for an immediate colour change. A positive reaction would

have given a muddy green colour whereas with negative reaction the broth remained yellow.

2.12. 4. Urease Production

The medium was Urea Agar Base (Oxoid Ltd.) prepared following the manufacturer's instruction to which 40% (v/v) sterile urea solution was added, aseptically dispensed into bijou bottles and contents allowed to set at an angle to prepare slopes. The medium was inoculated by heavily streaking the slope with the test organism, incubated at 37°C and examined daily for 5 days. A bright red or pink colouration indicated a positive reaction whereas a negative reaction was indicated by no change in the colour of the medium.

2.12. 5. Hydrogen Sulphide Production

The medium was prepared according to the formula in Cowan (1974) except that the sugars were omitted since these can interfere with the results obtained. The medium was dispensed in 3ml aliquots sterilized at 121°C for 20 min. sloped as described above and inoculated by stabbing the butt using a straight wire. The incubation temperature was 37°C with daily examination of the slopes for up to 5 days for blackening.

2.12. 6. Fermentation of Glucose and Inositol

Peptone water was the basal medium to which Andrade's indicator was added. The media were prepared as described by Cowan (1974). Glucose was sterilized by filtration and aseptically added to the sterile peptone water plus indicator. This was then distributed in 3ml aliquots into sterile bijou bottles with inverted Durham's tubes by employing full aseptic technique.

Inositol, the non-carbohydrate, was added to the peptone water base plus indicator, mixed, distributed in 3ml aliquots into bijou bottles and steamed for 30 min. The final concentration of glucose

and inositol in both media was 1%. Acid formation and gas production was recorded as stated earlier.

2.12.7. ONPG Test

The ONPG broth (Lowe, 1962) was prepared following the instructions in Cowan (1974) dispensed in 2ml aliquots into sterile bijou bottles and stored at 4°C. The broth was inoculated with a loopful of colonies of the test organism removed from an agar plate and incubated at 37°C overnight. A positive reaction was indicated by a yellow colour whereas with a negative reaction the broth remained colourless.

2.12.8. Gelatin Liquefaction

Nutrient gelatin (Oxoid Ltd.) was made up, dispensed in 10ml aliquots in Universal bottles and sterilized by autoclaving at 121°C for 20 min. This was then inoculated by stabbing and incubated at 30°C for 7 days with daily examination. To detect liquefaction, the bottles were placed in an ice-bucket for 30 min and observed for liquefaction. An uninoculated control was set up in parallel.

2.12.9. Oxidase Test

A filter paper was placed in a petri dish and was saturated with the reagent(2.2). Using a wooden toothpick, a portion of the colony to be tested was picked and rubbed on to the filter paper. A positive result would have been indicated by a dark purple colour appearing within 10 sec. whereas there was no change in the colour of the reagent with a negative result.

2.12.10. Catalase Test

The test organism was grown on a nutrient agar slope and after overnight incubation at 37°C, 1ml of a 3% (v/v) hydrogen peroxide solution was allowed to flow over the surface of the slope and examined immediately. The evolution of gas bubbles was regarded as a positive result: in a negative result no gas bubbles were produced.

2.12.11. Motility

Motility was examined by the hanging drop technique on an early exponential phase broth culture using a high power dry objective and reduced illumination.

2.12.12. Additional Diagnostic Tests

(a) Deoxyribonuclease (DNase) Test

The medium (Jeffries et al., 1957) was prepared as described in Cowan (1974). The medium was inoculated heavily on one spot and incubated at 37°C for 36h. The plate was flooded with 1N.HCl, excess acid tipped off and examined. A clear zone around the growth was regarded as a positive result. There was no clearing in a negative result.

(b) Tween 80 Hydrolysis

The medium used was that described by Lovell and Bibel,(1977). The medium was inoculated as above and incubated at 37°C for 48h. An opaque halo around the growth was recorded as a positive result. No halo around growth indicated a negative result.

2.13. Confirmatory Test - The API 20E Enterobacteriaceae System

This is a commercially available rapid multiple test system for the identification of the Enterobacteriaceae. The identification of an unknown species is based on the calculation of the likelihood between the unknown profile and each of the 50 species or subspecies stored in the memory bank of a computer, the unknown being identified as the species with the greatest likelihood. Likelihood values are translated into estimated frequencies of occurrence. The Analytical Profile Index lists over 40,000 bacterial strains. The nomenclature used throughout the index is that employed by the U.S. Department of Health, Education and Welfare Center for Disease Control. The strip was inoculated as directed by the manufacturer, incubated at 37°C for

between 18 and 24h, where appropriate, reagents were then added and the results recorded. A colour coder converted the colours given by the tests into a seven digit API Profile Index and this number was then found in the API Computer data book which gave the most probable identity of the organism.

2.14. Group D Streptococci Characterization Test Procedures

The tests described below were those deemed most appropriate and were selected from various publications (Facklam, 1972; Cowan, 1974; Gross et al. 1975; Parker and Ball, 1976). The expected results for the tests used in this investigation are shown in Appendix D (p.168).

2.14.1. Haemolytic Activity

All isolates were grown overnight at 37°C on blood agar plates (Blood Agar Base No.2 (Oxoid Ltd.))supplemented with 7% (v/v) sterile Defibrinated Horse Blood (Gibco Europe). Haemolysis was defined according to the appearances of well isolated colonies on the blood agar plates as: Alpha (α): a distinct zone of partial destruction of the red blood cells accompanied by a greenish discolouration of the medium. Beta (β): complete clearing and colourless zone around the colony in which the red blood cells have undergone complete discolouration, and Gamma (γ): no apparent haemolytic action or discolouration produced by the colony.

2.14.2. Growth at 10°C and 45°C

The medium used was Brain Heart Infusion broth (Oxoid Ltd.) 5ml of the sterile broth was inoculated and incubated in a thermostatically controlled cold temperature incubator set at 10°C for the 10°C test. For testing growth at 45°C, the broth was incubated in a water bath set at that temperature. The cultures were examined daily for 5 days. Growth was indicated by the turbidity of the broth.

2.14.3. 6.5% (w/v) NaCl Tolerance

Brain Heart Infusion broth (Oxoid Ltd.) was the basic medium.

It was made up with sodium chloride to a final concentration of 6.5% (w/v) NaCl. Immediately after inoculation, the medium was incubated at 37°C for 48h. Tolerance of this concentration of NaCl was indicated by the turbidity of the broth.

2.14.4. 0.04% (w/v) Potassium Tellurite Tolerance (Skadhauge, 1950)

The basal medium was Brain Heart Infusion Agar (Oxoid Ltd.) After autoclaving, the medium was cooled to 50°C in a thermostatically controlled water bath. 10ml of a 4% (w/v) potassium tellurite solution, previously sterilized by filtration, was added to one litre of the basal medium, mixed and 50ml sterile defibrinated horse blood added. After further mixing the medium was poured into petri dishes and allowed to set and dried. The plates were inoculated with the test organisms, streaked out to obtain well isolated colonies and incubated at 37°C for up to 3 days. Tellurite tolerance was recorded as positive during daily examination when the plates revealed jet-black or grey colonies.

2.14.5. Reduction of 0.1% (w/v) Tetrazolium (Barnes, 1956)

The basic medium was Brain Heart Infusion agar adjusted to pH 6.0 with acid, sterilized and cooled to 50°C. A 1% (w/v) solution of 2:3:5-triphenyltetrazolium chloride (sterilized by filtration) was added to a final concentration of 0.1% (v/v). The medium was poured into one half of two-compartment petri dishes (Sterilin Ltd.) whilst the other half contained blood agar. The test organisms were cross-streaked, starting from the blood agar and right across the plate to the tetrazolium end, incubated at 37°C and read daily for 3 days. Reduction of tetrazolium was revealed by brick-red colonies on examination.

2.14.6. Growth on 10% and 40% Bile-Aesculin Media (BAeM)

A two-compartment petri dish in which the medium in one half

was blood agar and in the other half was nutrient agar (Oxoid Ltd.) containing (a) Ox-Bile-Desiccated (Oxoid Ltd.) to a concentration equivalent to 10% (w/v) or 40% (w/v) of bile, (b) aesculin 0.1% (w/v) and (c) ferric citrate (0.05% (w/v)). As the aesculin was difficult to dissolve, it was heated mildly and stirred into the medium before autoclaving. The test organisms were cross-streaked as previously described and incubated at 37°C for 48h. Bile tolerance was indicated by growth on both media and aesculin hydrolysis by growth right across the plate with blackening of the bile-aesculin medium due to the conversion of aesculin to aesculitin detected by the ferric chloride.

2.14.7. Reduction of Tatrazolium and Decarboxylation of Tyrosine (Mead, 1963).

The medium was prepared as described (D.H.S.S. (Welsh Office), 1969). The medium was inoculated with the test organism and streaked out to obtain well isolated colonies. It was incubated at 45°C for 3 days with daily examination. The concomitant reduction of the tetrazolium and the decarboxylation of tyrosine produced distinctive red metallic sheen colonies with translucent zones around them.

2.14.8. Hydrolysis of Starch

The procedure recommended by Cowan (1974) was used. The starch-agar plate was inoculated heavily on one spot and incubated at 37°C for 4 days. The plate was flooded with Lugol's iodine and excess iodine drained off. Hydrolysis of the starch was indicated by a clear, colourless zone around the spot inoculum. Where the starch has not been hydrolysed the medium turned blue.

2.14.9. Liquefaction of Gelatin

The medium was the same as described previously and the same procedure was followed.

2.14.10. Apparent Pigment Production

The medium was that of Jones et al. (1963). The test organisms were streaked out on the medium so as to obtain discrete colonies and incubated aerobically at 37°C for 48h and examined for brown colonies.

2.14.11. Pigment Production

Pigment production was determined using both blood agar plates and Brain Heart Infusion agar plates supplemented with 5% (w/v) sucrose. Yellow colonies on both media were recorded.

2.14.12. Catalase Production

The isolates were grown on glucose yeast extract agar slopes overnight and tested as described previously.

2.15. Carbohydrates for Group D Streptococci Fermentation Tests

2.15.1. Basal Medium

For all the fermentation activity tests, except glycerol, the basal medium to which the appropriate sugar was added to give a final concentration of 1% (w/v) contained:

Peptone (Oxoid Ltd.)	10g
NaCl	5g
K_2HPO_4	1g
Distilled water to	1000ml

The ingredients were dissolved by heating in the water and the pH adjusted appropriately between pH 7.2 and pH 7.4.

2.15.2. Indicator Solution

The indicator solution was prepared by dissolving 0.2g bromocresol purple in 50ml ethanol. The solution was then made up to 100ml with distilled water.

2.15. 3. Carbohydrate Solutions

10g of the appropriate sugar (see below) was dissolved in 90ml of distilled water. Some sugars were autoclaved with the basal medium, others which are heat-labile were filter-sterilized separate from the basal medium. Those sugars that were autoclaved or filter-sterilized are listed below.

2.15. 4. Carbohydrates Sterilized by Autoclaving

These were:- mannitol
sorbitol
inulin
lactose

2.15.5. Carbohydrates Sterilized by Filtration

These were:- arabinose
melezitose
melibiose

2.15. 6. Preparation of Sterilizable Sugars

90ml of the appropriate sugar solution was added to 900ml of the basal medium to which 10ml of the indicator solution was added. This was thoroughly mixed and dispensed in 3ml aliquots into bijou bottles and autoclaved at 115°C for 20 min.

2.15. 7. Preparation of Non-Sterilizable Sugars

900ml of the basal medium plus 10ml of the indicator solution was sterilized at 115°C for 20 min and 90ml of the appropriate filter-sterilized sugar solution was aseptically added and aseptically dispensed in 3ml aliquots into sterile bijou bottles.

2.15. 8. Anaerobic Fermentation of Glycerol

The medium contained:	Peptone (Oxoid Ltd.)	10g
	Yeast extract (Oxoid Ltd.)	10g
	Glycerol to 0.5% (v/v)	
	Distilled water	990ml

The peptone and yeast extract were dissolved in 990ml water and 4ml of the indicator solution (resazurin:10mg in 40ml distilled water) added and mixed. 6.3ml of glycerol was added to this to

give a final concentration of 0.5% (v/v) mixed, dispensed in 3ml aliquots into bijou bottles and sterilized at 115°C for 20 min. The yeast extract in the medium acts as a hydrogen acceptor (Gunsalus, 1947), the resazurin is an Eh indicator. Just prior to inoculation, the bottles were steamed to drive off oxygen, immediately inoculated and layered with sterile mineral oil (liquid paraffin) and incubated at 37°C for 7 days with daily examination.

2.15. 9. Arginine Hydrolysis

The medium was arginine agar (Thornley, 1960) and contained:

Peptone (Oxoid Ltd.)	1g
NaCl	5g
K_2HPO_4	0.3g
Phenol red	0.01g
L-arginine menohydrochloride	10g
Agar	3g
Distilled water to	1000ml

The solids were dissolved by heating and the pH adjusted to 7.2. The medium was distributed in 3ml aliquots into bijou bottles and sterilized. The medium was inoculated with the test organism by stabbing using a straight wire and immediately overlayered with sterile liquid paraffin and incubated at 37° for 7 days with daily examination. A positive reaction gave a red colouration.

2.15. 10. Pyruvate Fermentation

The medium was that of Gross et al., (1975) and contained:

Tryptone (Oxoid Ltd.)	10g
Yeast extract (Oxoid Ltd.)	5g
K_2HPO_4	5g
NaCl	5g
Sodium salt of pyruvic acid	10g
Bromothymol blue	0.04g
Distilled water to	1000ml

The solids were dissolved by heating and the pH adjusted to between 7.2 and 7.4. The broth was dispensed into bijou bottles in 3ml aliquots and autoclaved at 115°C for 15 min. The medium was inoculated and incubated at 37°C overnight. A positive reaction was given by a bright yellow colour whereas with a negative reaction the medium remained blue-green.

2.16. Group D Reaction

The coagglutination method described below was used in the detection of the group D antigen in the streptococci.

2.16.1. Reagent Staphylococci Preparation

Materials

Casamino-casein hydrolysis yeast extract (CCY) broth contained:

Bacto casamino acid (Difco)	30.0g
Casein hydrolysate (Oxoid)	29.3g
Yeast extract (Oxoid)	5.0g
Distilled water	1000ml

The ingredients were dissolved in the water, the pH adjusted to 6.8, distributed in 500ml aliquots and autoclaved at 110°C for 10 min.

2.16.2. Phosphate Buffered Saline (PBS)

NaCl	8.0g
K_2HPO_4	1.21g
KH_2PO_4	0.34g
Distilled water	1000ml

The solids were dissolved in the water, the pH measured 7.3 and sterilized by filtration.

2.16.3. Formalin - P.B.S.

Formaldehyde was added to the buffered saline to a concentration of 2% (v/v).

2.16.4. Azide P.B.S.

Sodium azide was added at a concentration of 0.1% to the buffered saline.

2.16.5. Reagent Staphylococci

The method described below was adapted from (Christensen et al. 1973). Cowan 1 strain Staphylococcus aureus NCTC 8530 was grown in 500ml CCY broth overnight in an orbital incubator-shaker at 37°C. The cells were harvested by centrifugation at 6000 rpm for 10 min and washed twice with buffered saline.

2.16.6. Formalization of the Cells

The pellet was resuspended in 5 times the packed cell volume of formalin buffered saline and stirred overnight at 4°C. The cells were washed again in buffered saline, centrifuged as before and the pellet resuspended in 3 times the packed cell volume in buffered saline.

2.16.7. Heat Stabilization of the Cells

The suspension was heated in a water bath at 80°C for 5 min and transferred to an aluminium vessel. The vessel was submerged in oil bath at 80°C for a few seconds and cooled rapidly on dry ice. The cells were resuspended in buffered saline and washed twice again. The cell volume was measured and azide-buffered saline added to a final concentration of 10% (v/v), distributed in 10ml aliquots and stored at 4°C until used.

2.16.8. Coating of Staphylococcus aureus

Materials

10% heat-stabilized staphylococcal suspension
PBS
Azide-PBS
Group D (Streptococcus Grouping Serum rabbit)
(Wellcome Reagents Ltd.)

0.1ml of group D antiserum was added to 1ml of 10% staphylococci suspension mixed and washed twice with PBS. The packed cells were

resuspended in azide-PBS to give a final cell concentration of 1% (v/v).

2.16.9. Trypsinization of the Streptococci

Materials

0.2M Tris pH 7.8
Trypsin (Sigma) (5mgml^{-1} solution in 0.2M Tris)
Blood agar base No.2 (Oxoid Ltd.)
Sterile defibrinated horse blood

Pure cultures of the streptococci strains to be tested were obtained by growing the strains overnight at 37°C on blood agar. Heavy suspensions of the strains were made in 0.5ml Tris pH 7.8 and 0.1ml of the trypsin solution added and incubated at 37°C for 1h in a water bath.

2.16.10. Grouping by Coagglutination

A drop of the trypsinized streptococcus was placed on a clean microscope slide, to this was added a drop of the coated staphylococcal suspension, mixed and rocked gently and observed for clumping. Coagglutination usually occurred within 30 secs to 1 min.

2.17. The Rationale of the Selection of the Antibacterial Agents used in this Investigation

With the exception of streptomycin, the selection of the antibacterial agents used in this study was restricted to drugs known to be therapeutically useful and likely to be prescribed in the local community or administered in a clinical environment.

Streptomycin, one of the earliest drugs used, was included in the list because a high percentage of microorganisms are known to be resistant to this agent. These agents, except streptomycin, are thought to be appropriate in the treatment of common bacterial infections such as those of the urinary tract and upper respiratory

tract infections. Selection of these agents was also influenced by the type of bacteria that were isolated and their susceptibilities to these drugs with reference to publications in the literature.

2.17.1. Antibacterial Agents - Sources

Wherever possible, the standard antibacterial agents used were specifically for susceptibility testing and were obtained from the manufacturers. The drug samples bore a label stating the activity of the agent expressed in micrograms per milligram and an expiry date. The drugs used were ampicillin (Ap) (Beecham Res. Labs.), streptomycin sulphate (Sm) (Sigma), chloramphenicol (Cm) (Sigma), sulphamethoxazole (Su) (Burroughs Wellcome), tetracycline (Tc) (Sigma), cephalexin (Cx) (Lilly), trimethoprim lactate (Tm) (Burroughs Wellcome), benzyl penicillin (Pn) (Glaxo), gentamicin sulphate (Gm) (Sigma) and erythromycin (Em) (Sigma). The antibacterial agents were stored at $4^{\circ}C$.

2.17.2. Preparation of Antibacterial Agents - Stock Solutions

The powders were weighed on an analytical balance and dissolved in the appropriate solvent(s) (Table 2.1) to yield the required concentration of the active drug per millilitre. The concentration was determined by relating it to the activity standard given by the manufacturer.

Even though many antibacterial agents may be dissolved in distilled water, some require special solvents or pH adjustments for the initial solubilization. Table 2.1 lists the solvents, buffer solutions and diluents used.

The stock solutions (usually $1000 \mu g.ml^{-1}$) were stored frozen at $-20^{\circ}C$ in aliquots in individually tightly sealed containers. When required they were removed, thawed, diluted and used within

TABLE 2.1. SOLVENTS AND DILUENTS FOR STOCK SOLUTIONS PREPARATION OF ANTIBACTERIAL AGENTS

(Garrod et al., 1973; Lennette et al., 1974)

Ampicillin	0.2M Phosphate buffer pH 8.0	0.2M Phosphate buffer pH 8.0
Cephalexin	0.2M Phosphate buffer pH 8.0	0.2M Phosphate buffer pH 8.0
Chloramphenicol	Ethanol	Water
Erythromycin	Ethanol	Water
Fusidic acid	Water	Water
Gentamicin	0.2M Phosphate buffer pH 8.0	Water
Kanamycin	0.2M Phosphate buffer pH 8.0	Water
Nalidixic acid	1N NaOH	Water
Neomycin	0.2M Phosphate buffer pH 8.0	Water
Penicillin	Water	Water
Rifampin	Dimethyl sulphoxide	0.2M Phosphate buffer pH 7.0
Streptomycin	0.2M Phosphate buffer pH 8.0	Water
Sulphamethoxazole	Hot water + minimal amount of 10% NaOH to dissolve	Water
Tetracycline	Ethanol or 0.01N HCl	0.2M Citrate-phosphate buffer pH 4.5
Trimethoprim	0.2M Citrate-phosphate buffer pH 5.0	Water
Vancomycin	Water	Water

a day and were never refrozen. Unused material that had been thawed was discarded. Most stock solutions of antibacterial agents remain stable at -20°C for up to three months. However, ampicillin does not retain at least 90% of its potency for long periods (Warren et al., 1972).

2.17.3. Upper and Lower Limits of Drug Concentration

Table 2.2 (a and b) lists the upper and lower limits of drug concentration used for the susceptibility testing of the isolates. These limits were considered satisfactory based on data obtained from Garrod et al.(1973). The ranges chosen also included the end-points of the reference strains to permit adequate control of the method.

2.18. Minimum Inhibitory Concentration (MIC) Determination

A range of doubling concentrations of the antibacterial agents in the appropriate diluent was prepared at 10 times the final concentrations required in the agar. Diagnostic Sensitivity Test Agar (DST)(Oxoid Ltd.) was used. 10ml of the appropriate dilution of the agent was added to 90ml sterilized, cooled (50°C) agar and well mixed. The antibiotic agar was poured (25ml) into 4 previously labelled petri dishes and allowed to set at room temperature. The plates were dried in an LTE Edmunds oven set at 37°C with the lids tipped. Whenever possible the plates were used immediately or were stored at 4°C and used within 5 days.

The activity of the sulphonamides and trimethoprim is partly antagonised by the components of all bloods except lysed horse blood (Garrod et al., 1973). For this reason, the DST agar was supplemented with 4% (v/v) saponin-lysed horse blood for the testing of Sulphamethoxazole and trimethoprim. For the in vitro

TABLE 2.2 UPPER AND LOWER LIMITS OF ANTIBACTERIAL AGENTS USED FOR SUSCEPTIBILITY TESTING.

(a) For the Testing of Coliform Isolates

Antibacterial agents	Range of concentrations ($\mu g\ ml^{-1}$) Lower limit	Upper limit
Ampicillin	1	32
Cephalexin	1	64
Streptomycin	1	64
Tetracycline	1	64
Sulphamethoxazole	1	64
Chloramphenicol	1	64
Trimethoprim	0.18	10

(b) For the Testing of Group D Streptococci Isolates

Ampicillin	0.03	16
Benzylpenicillin	0.03	16
Streptomycin	64	1000
Gentamicin	8	256
Erythromycin	0.03	1000
Tetracycline	0.25	1000

susceptibility testing of the group D streptococci isolates, the agar was supplemented with 4% (v/v) defibrinated horse blood.

For the whole period of the susceptibility testing, the DST agar was sterilized and cooled to 50°C suspended in a water bath to equilibrate the medium prior to the addition of the agent. If the antibacterial agent were to be added when the medium was at higher temperatures, this would cause the deterioration of the agent; addition of the agent at lower temperatures would preclude thorough and adequate mixing.

2.18.1. Inocula Preparation

Five well isolated colonies on a solid medium were touched with a loop and emulsified into 3 ml sterile Brain Heart Infusion broth and incubated at 37°C overnight. A one in a hundred dilution of the overnight broth culture was made to give a bacterial density of 10^5 to 10^6 colony-forming units ml^{-1} (Fass and Prior, 1978).

2.18.2. Application of Inocula and Incubation

All inocula were applied with a multiple inoculator to all drug plates. Control plates with no drugs were set up as well as reference strains with known MIC's. The plates were incubated overnight at 37°C and examined the following day. The M.I.C. for each drug was recorded as the lowest concentration ($\mu g.ml^{-1}$) on which five or less colonies grew.

2.18.3. Determination of Resistance in the Coliforms

So far as determination of strain resistance in the coliform was concerned, growth in the presence of a wide range of concentrations of individual drugs had been used by different workers, and particularly where clinical isolates were involved. In this investigation, bacteria were deemed resistant if they had an M.I.C. value equal to or greater than the drug concentration values ($\mu g.ml^{-1}$) as shown in Table 2.3(a).

TABLE 2. 3.(a) MINIMUM INHIBITORY CONCENTRATION VALUES[1] ($\mu g\ ml^{-1}$)
OF WHICH THE COLIFORM GENERA WERE DEEMED TO BE RESISTANT

	Ap	Su	Cx	Tc	Sm	Cm	Tm
E. coli	16	16	16	8	4	16	3
Enterobacter spp.	32	32	32	32	8	16	3
Klebsiella spp.	32	32	16	8	8	16	1.5
Serratia spp.[2]	32	32	32	8	8	16	4.5
Citrobacter spp.	16	16	16	8	8	16	0.75

[1]The values taken were in accord with previous reports (Andrews *et al.* 1975; Bushby, 1973; Fass and Prior, 1978; Garrod *et al.* 1973; Kelch and Lee, 1978; Lindin-Janson *et al.* 1977; Linton *et al.* 1974; McGowan *et al.* 1974; Shah *et al.* 1979; Slocombe *et al.* 1975).

[2]No *Serratia marcescens*, which tend to have higher M.I.C. values than other Serratia species, were identified. Most of the *Serratia* isolates were identified as *Serratia liquefaciens*. Until recently these were classified with the *Enterobacter* species and so these values were chosen bearing this in mind.

TABLE 2.3.(b) ANTIBIOTIC SENSITIVITY GROUPING OF GROUP D STREPTOCOCCI
M.I.C. (μgml^{-1}) RANGE OF GROUP LIMITS

Antibiotic	1. Sensitive	2.Mod.resistant	3.Resistant
Ampicillin	$\leq$0.03 - 1.0	2.0 - 16	>16
Benzylpenicillin	$\leq$0.03 - 2.0	4.0 - 16	>16
Streptomycin	16 - 64	128 - 1000	>1000
Gentamicin	1.0 - 8	16 - 64	>64
Erythromycin	$\leq$0.03 - 2.0	4.0 - 16	>16
Tetracycline	$\leq$0.03 - 0.5	1.0 - 16	>16

2.18. 4. Determination of Resistance in the Group D Streptococci

While there is a great deal of information on the susceptibility of coliforms, little is available about group D streptococci. The examination of available information revealed that these streptococci were all grouped under the broad heading of enterococci without reference to the different species (or variants of a species) within the group. The susceptibility of different species of human isolates of group D streptococci has been studied by (Toala et al. 1969; Wilkowske et al. 1974; Thornsberry et al. 1974). When the MIC's obtained from these studies were compared with results obtained from this investigation, there was a general agreement, as a result Table 2.3(b) was drawn to group these organisms.

2.19. Statistical Evaluation

With respect to the isolates of each species or genus from above and below the sewer outfall, a test of the null hypothesis of equal proportions resistant and sensitive to a given drug against a two-sided alternative was made using the Chi-square approximation with continuity correction. In the cases where Chi-square approximation was not valid, Fisher's Exact Test was used. In this case, the test was against a one-sided alternative hypothesis.

2.20. Isolation of Nalidixic Acid Resistant Mutants

MacConkey agar (Oxoid Ltd.) plates containing varying concentrations of nalidixic acid, ranging from $5 \mu g ml^{-1}$ to $320 \mu g ml^{-1}$ were prepared.

Nalidixic acid-sensitive strains of coliforms, to be used as recipients when the donors were gram-negative bacilli, were grown overnight at $37^{o}C$ in Brain Heart Infusion broth. 0.1ml of the overnight broth culture of each strain was directly spread over the entire surface of the antibiotic agar containing the lowest

concentration of the drug and incubated overnight at 37°C. Stepwise increase in resistance was obtained by the transfer of colonies that developed on to fresh plates containing a higher concentration of the drug. The mutant cells were passaged up to the top concentration of the drug (Table 2.4). A fusidic acid and rifampin-resistant mutant of S. faecalis was obtained from Dr. van Embden for conjugation studies between group D streptococci.

2.21. Conjugation Procedures

Tables 2.5(a and b) list details of the organisms that were used for the conjugation studies. These were all selected on the basis that they displayed resistance to at least three of the antibacterial agents against which they were tested.

Materials Used

Nutrient broth No.2 (Oxoid Ltd.)
Blood agar base No.2 "
DST agar "
MacConkey agar "
Stock solutions of the following antibacterial agents.
Ap, Pn, Cx, Sm, Tc, Em, Nal and Fusidic acid (Fn).

2.21.1. Selection Plates

The concentration of antibacterial agents in the medium for the selection of plasmid transfer was the lowest necessary to completely inhibit the sensitive recipient. For the resistance transfer studies between coliform isolates, the selection plates for Ap, Cx, Sm and Tc transfer, were MacConkey agar (Oxoid Ltd.) plates containing nalidixic acid at 10μg.ml^{-1} which completely inhibited the donor strains. In addition, drugs to which the donor strains were resistant were added separately and in combination, each at a concentration of 10μg.ml^{-1}. R^+ recipients were distinguished from mutants of the donors by their lactose fermentation reaction. For the selection of sulphamethoxazole

TABLE 2.4. NALIDIXIC ACID-RESISTANT MUTANTS USED FOR INTERGENERIC CONJUGATION STUDIES

STRAIN	RELEVANT DETAILS	SOURCE
CB95-1	*Citrobacter freundii*, natural isolate, mutant of CB95, resistant to nalidixic acid, sensitive to ampicillin.	The Author
CB86-1	*Enterobacter cloacae*, natural isolate, mutant of CB86, resistant to nalidixic acid, sensitive to ampicillin.	"
CA53-1	*Klebsiella species*, natural isolate, mutant of CA53, resistant to nalidixic acid, sensitive to ampicillin.	"
CB32-1	*Serratia liquefaciens*, natural isolate, mutant of CB32 resistant to nalidixic acid, sensitive to ampicillin.	"
NICB 9482-1	*E. coli* K-12, mutant of 9482, resistant to nalidixic acid and streptomycin.	NICB
NICB 9484-1	*E. coli* K-12, mutant of 9484, resistant to nalidixic acid sensitive to streptomycin.	"

TABLE 2.5.(a) COLIFORM STRAINS USED FOR CONJUGATION STUDIES

STRAIN		RELEVANT DETAILS		SOURCE
CB18	*E. coli*	natural isolate, resistant to	Ap Sm and Cx	The Author
CA22	*Enterobacter species*	"	Ap Su and Cx	"
CB1	*Citrobacter species*	"	Ap Sm Su	"
CB13	*E. coli*	"		"
CB17	*E. coli*	"		"
CB24	*E. coli*	"		"
CB80	*E. coli*	"		"
CA54	*E. coli*	"	Sm Tc and Cx	"
CB6	*E. coli*	"	Sm Su Cx	"
CB9	*E. coli*	"		"
CB31	*E. coli*	"		"
CB50	*E. coli*	"		"
CB62	*Serratia species*	"		"
CB77	*E. coli*	"		"
CB81	*Enterobacter species*	"		"
CA1	*E. coli*	"		"
CA5	*E. coli*	"		"
CA34	*E. coli*	"		"
CA35	*E. coli*	"		"

TABLE 2.5. (a) (Contd.) COLIFORM STRAINS USED FOR CONJUGATION STUDIES

	STRAIN	RELEVANT DETAILS		SOURCE
CB25	E. coli	natural isolate, resistant to		The Author
CB27	E. coli	"		"
CB34	E. coli	"		"
CB38	E. coli	"	Sm Tc Su	"
CB43	E. coli	"		"
CB49	E. coli	"		"
CB74	E. coli	"		"
CB3	E. coli	"		"
CB14	E. coli	"		"
CB28	E. coli	"		"
CB61	E. coli	"	Ap Sm Su Cx	"
CB88	E. coli	"		"
CB89	E. coli	"		"
CB97	E. coli	"		"
CA31	E. coli	"		"
CB51	E. coli	"		"
CB93	E. coli	"	Sm Tu Su Cx	"
CA43	E. coli	"		"
CA67	E. coli	"		"
CA93	E. coli	"	Ap Sm Tc Su Cx	"

TABLE 2.5.(b) DRUG RESISTANT GROUP D STREPTOCOCCI USED FOR CONJUGATION STUDIES

STRAIN		RELEVANT DETAILS	SOURCE
JH2	S. faecalis	non-haemolytic	Jacobs and Hobbs, 1974
JH2-2		mutant of JH2, resistant to Fn and Rif	"
DS5	S. faecalis	natural isolate, β-haemolytic, resistant to Tc and Em	Clewell et al. 1974
SB69	S. faecium	" α-haemolytic, " Ap Pn Sm and Tc	The Author
SB94	S. faecalis	" non-haemolytic, " Sm Em and Tc	"
K46	S. faecium	clinical isolate, α-haemolytic, resistant to Ap Pn Sm Em Tc	Barrie, 1979
K55	S. faecalis	clinical isolate, non-haemolytic, resistant to Sm Em and Tc	"
K60	S. faecalis var. liquefaciens	" Sm Em and Tc	"
K87	S. faecalis	var. zymogenes clinical isolate, β-haemolytic, resistant to Sm Em and Tc	"
K88	S. faecalis	var. liquefaciens clinical isolate, non-haemolytic, resistant to Sm Em and Tc	"

resistance transfer between coliform isolation, DST agar (Oxoid Ltd.) plates were used supplemented with 4% saponin-lysed defibrinated horse blood to which nalidixic acid ($10\mu g.ml^{-1}$) and sulphamethoxazole ($10\mu g.ml^{-1}$) had been added.

Blood agar base No.2 (Oxoid Ltd.) plates supplemented with 7% (v/v) saponin-lysed horse blood was the medium for selection plates for resistance transfer between the group D streptococci isolates. The medium contained fusidic acid at $25\mu g.ml^{-1}$ to which the donor strains were sensitive, plus one of the following drugs: Ap, Pn, Sm, Em or Tc. The actual concentration of the drugs used were Ap $2.5\mu g.ml^{-1}$, Pn $2.5\mu g.ml^{-1}$, Tc $25\mu g.ml^{-1}$, Em $25\mu g.ml^{-1}$ and Sm $100\mu g.ml^{-1}$.

2.21.2. Conditions for Transfer

(a) Donor Strains. These were grown without aeration to late exponential phase in nutrient broth to approximately 2×10^8 organisms ml^{-1}.

(b) Recipient Strains. The recipient strains were grown aerobically by shaking in a New Brunswick G25 orbital incubator-shaker to late exponential phase at 37°C to approximately 5×10^8 organisms ml^{-1}. They were then diluted with an equal volume of fresh warm nutrient broth.

2.21.3. Mating Mixture

One volume of the donor strain was mixed with 9 volumes of the recipient strain. The mixture was incubated at 37°C overnight without shaking. When investigating the frequency of transfer the mixture was incubated at 37°C for 4h with shaking.

2.21.4. Selection of transconjugants

The mating mixtures were agitated vigorously using a rota mixer to separate mating pairs and then 0.1ml of the undiluted overnight mixtures were spread evenly over one half of the selection plates (for the donor, recipient and the transconjugant) and streaked thinly over the other half so as to obtain single colonies, and incubated at 37°C overnight. For frequency of transfer studies, the 4h mixtures were agitated

vigorously, diluted down to 10^{-7} and 0.1ml of the appropriate dilutions were spread evenly over the entire surface of the selection plates and incubated at 37°C overnight. Individual colonies that developed on the selection plates were counted for frequency of transfer determination and were rechecked for purity by streaking onto a second drug-free plate to obtain single colonies. The pure cultures were then tested for the unselected trait of the recipient, and for their entire resistance pattern.

2.22. Curing Procedures

2.22.1. Curing Cells of Plasmids with Acridine Orange

Overnight cultures in Brain Heart Infusion broth of the strains to be cured were made. These were diluted 1 in 100 in fresh Brain Heart Infusion broth, acridine orange was added to a final concentration of $10\mu gml^{-1}$ or $25\mu gml^{-1}$. The former concentration was for group D streptococci plasmid curing and the latter for coliform plasmid curing. The cultures were incubated in the orbital incubator shaker overnight at 37°C. At the end of the incubation period the cultures were diluted to 10^{-5}. 0.1ml of a 10^{-4} dilution of the streptococcal cultures were spread evenly on blood agar plates and 0.1ml of a 10^{-5} dilution of the coliform cultures were spread on MacConkey agar plates which were incubated at 37°C overnight. Approximately 300 colonies on the plates were tested for resistance loss by replica plating (Lederberg and Lederberg, 1952) from the drug-free media to plates containing antibiotics. Colonies present on the master plates but absent on the replica plates were subcultured from the master plates to check the identity and drug sensitivity.

2.22.2. Curing at an Elevated Temperature

For curing at an elevated temperature the overnight broth cultures were diluted for streptococci and coliforms respectively as before, and 0.1ml of the appropriate dilutions were spread on blood and

MacConkey agar plates and incubated at 45°C overnight. Colonies developing on the plates were tested for resistance loss as described previously.

2.23. Isolation of Plasmids

Plasmid DNA can be isolated from normal cells harbouring drug resistance factors. The plasmid DNA is generally present in the cell as covalently closed circular DNA (cccDNA). Basically the procedure commonly adopted for their isolation involves lysis of the cell, removal of chromosome and debris, concentration of the remaining DNA and finally resolution of the plasmid DNA.

Lysis of the cell is brought about in two stages. First, spheroplasts are formed by digesting the rigid outer layer of the cell with the enzyme lysozyme (lysostaphin is used with staphylococci) in the presence of sucrose. The sucrose protects the cell from bursting by providing a high external osmotic pressure. Next, the spheroplasts are then lysed with detergent thereby releasing the DNA of both the plasmid and the chromosome.

A variety of methods are available to separate the plasmid DNA from the chromosomal DNA. Most of these methods are based on the covalently closed circular configuration of the plasmid. Because of their tightly twisted ring structure, they have decreased sensitivity to shear force, increase resistance to temperature and alkali denaturation. These characteristics allow the separation of the plasmid DNA from the chromosomal DNA. Many procedures involve a relatively slow and short centrifugation-clearing spin. This pellets most of the cell debris and the large chromosomal DNA. The smaller plasmid molecules usually contaminated with small pieces of chromosomal DNA remain in the supernatant-cleared lysate. Other procedures involve velocity gradient centrifugation where

plasmids often give two bands in a preformed gradient of sucrose solution, if the centrifugation is stopped before the DNA reaches the bottom of the tube. Plasmids usually give two bands because DNA can exist in two conformations, the covalently closed circle (ccc) or supercoil in which the molecule is tightly twisted, and the open circle (oc) where the supercoil is not maintained because of a nick in the supercoil.

Linear and open circular molecules can bind more of the intercalating compound ethidium bromide than covalently closed circular molecules. When this occurs in caesium chloride solution, caesium ions are displaced, and the DNA becomes less dense. Because of this difference in density closed circular molecules can be separated from other species of DNA on a caesium chloride-ethidium bromide density gradient. A comprehensive account on plasmid isolation methods is given by Broda (1979). Plasmid purification can be achieved by dialysis and an estimate of plasmid mass may be obtained by agarose gel electrophoresis.

The procedure adopted in the isolation of plasmid DNA is illustrated in the flow diagram below.

2.23.1. Materials for Plasmid Isolation

Stock solutions of chloramphenicol, tetracycline
Brain Heart Infusion broth (Oxoid Ltd.)
0.05M Tris pH 8.0 + 10% (w/v) sucrose
0.25M Tris pH 8.0
0.2M EDTA
2% (w/v) sarkosyl (N-lauroyl sarcosine, (Sigma)) in 0.05M Tris pH 8
Redistilled phenol
10mM Tris HCl pH 8
Isopropanol, Butan 1-ol
95% ethanol
Ethidium bromide (Sigma)
Caesium chloride (BRL)
Agarose (BRL)

TE buffer contained:

1M Tris pH 8	1ml
0.2M EDTA pH 8	0.5ml
Distilled water	98.5ml

Tracking Dye

Glycerol 50%
Xylene cyanol (Dye Eastman) 0.5%
Bromophenol blue 0.5%

TEA buffer

Tris base g	96.8
Sodium acetate g	54.4
Disodium EDTA g	14.49
NaCl g	21.04
Distilled water	2000ml

Adjusted to pH 8.5 with glacial acetic acid. Diluted 1 in 10 before use.

2.23. Plasmid Isolation Procedure

For a starter culture, cells were grown overnight at 37°C in 100ml Brain Heart Infusion broth in the presence of appropriate antibiotics.

This was used to inoculate 1 litre fresh Brain Heart Infusion broth and grown at 37°C with continuous shaking to an absorbance 650nm of 0.6-0.8. 2ml of a 10% (w/v) chloramphenicol solution (= 250μgml^{-1}) was added to amplify the plasmid. The culture was incubated overnight.

↓

Cells were spun down at 6000 rpm (8000g) for 10 min at 4°C.

↓

Pellets were resuspended in 120ml 0.05M Tris pH 8 + 10% (w/v) sucrose. The suspension was redistributed in 6 x 250ml conical flasks.

↓

4ml of freshly prepared lysozyme solution (5mgml^{-1} in 0.25M Tris, pH 8) was added and mixed again by hand swirling.

↓

The suspensions were left on ice bath for 5-10 min.

↓

4ml of 2% (w/v) sarkosyl was added and dispersed immediately. The suspensions became viscous and clear.

↓

The suspensions were centrifuged at 20,000 rpm (20,000g) for 2½h and the supernatants were removed.

↓

150ml of redistilled phenol, saturated with 10mM Tris, pH 8 were added and gently mixed and left on ice for 10 min.

↓

The suspensions were centrifuged at 8 000 rpm (10,000g) for 10 min and the aqueous layer removed.

↓

Twice the volume of cold (-20°C) 95% ethanol was added to precipitate the plasmid. The suspensions were left at -20°C overnight.

↓

The suspensions were centrifuged at 12,000 rpm (18,000g) for 10 min and the supernatants discarded. The tubes were inverted on absorbent kimwipes to allow the ethanol to drain off.

↓

The pellets were resuspended in 4ml T.E. buffer.

↓

Caesium chloride and ethidium bromide were added to the preparation which was then centrifuged at 38,000 rpm (45,000g) for 60h at 20°C to form a caesium chloride density gradient.

↓

The tubes were removed and viewed under an UV source emitting maximally at 302nm.

↓

The tubes were pierced from below and fractions containing plasmid bands were collected.

↓

The ethidium bromide was removed by adding butan 1-ol. After centrifugation the upper butanol layer containing ethidium bromide was removed and discarded.

↓

The fractions were dialysed against T.E. buffer overnight.

↓

The pure plasmid preparations were subjected to electrophoresis in 0.8% (w/v) slab agarose gels.

2.24. Isolation Procedure for Anaerobes

Figure 2.2 shows the sampling locations. The sampling procedure was as previously described. Samples of raw sewage liquor were collected on three separate occasions from the initial primary settling tank as the sewage entered the plant, and on one occasion, a sample was collected from the humus settling tank at the effluent end before the treated sewage was discharged as final effluent. Screw capped bottles containing anaerobic diluting fluid (Bryant and Burkey, 1953) were prepared and sterilized by filtration. These were used to prepare serial decimal dilutions down to 10^{-8} before filtration. For the filtration, gridded Nuflow membranes (Oxoid Ltd.), sterilized as described before, were used. 10ml aliquots from each of the dilutions 10^{-5} - 10^{-8} inclusive were filtered in quadruplicate. The membranes were layered onto the surfaces of well dried selective plates (May and Baker, 1979).

2.24. 1. Selective Plates

The basic medium was Brain Heart Infusion agar (Oxoid Ltd.) supplemented with yeast extract (Oxoid Ltd.). 0.05% (w/v) cysteine hydrochloride (Sigma) - 0.1% (w/v); and sterile defibrinated horse blood - 10% (v/v). The selective media given below were used. They were prepared by first of all autoclaving the basic medium with its supplements without the blood. The selective agent and the blood were added after the medium had been cooled to 50°C.

(i) neomycin (Nm) blood agar contained 100μgml^{-1} Nm (Upjohn Ltd.)

(ii) kanamycin (Km) blood agar contained 75μgml^{-1} Km (Sigma)

(iii) Km. Vancomycin (Vm) blood agar contained 100μgml^{-1} Km and 7.5μgml^{-1} Vm (Sigma)

(iv) Km-bile blood agar contained 100μgml^{-1} Km and 2% ox-gall (Oxoid Ltd.) (= 20% bile)

(v) Nm-Vm blood agar contained 100$\mu g.ml^{-1}$ Nm and 75$\mu g.ml^{-1}$ Vm

(vi) Rifampin (Rif) blood agar contained 50$\mu g.ml^{-1}$ Rif (C.P. Labs.)

In addition, samples from the primary settling tank only were grown on Perfringens agar (Oxoid Ltd.)

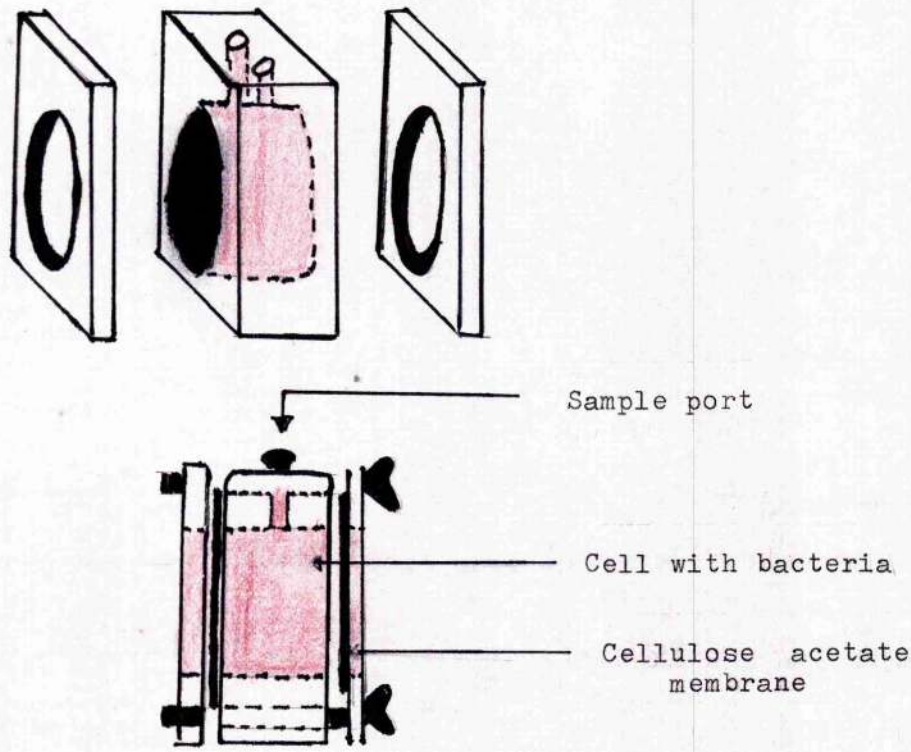
2.24.2. Incubation

With respect to each set of four selective plates, two were incubated in an atmosphere of 10% CO_2 at 37°C for the enumeration of microaerophiles. One of this pair of plates was incubated for 48h and the other for 5 days. For the enumeration of strict anaerobes two Fildes-McIntosh jars were set up, each containing one of the remaining pair of selective plates.

2.24.3. Evacuation and Replacement of Anaerobic Jars

Selective plates were placed in the respective Fildes-McIntosh jars. The lids of the jars were secured and one valve connected to a mercury manometer while air was evacuated through the other until the pressure within the jar drew a vacuum of 25 in (625mm Hg). The latter valve was then closed and the air replaced with an 80% N_2/10% H_2/10% CO_2 gas mixture (BOC Ltd.) through a football bladder until atmospheric pressure was restored. The valves were closed and the jars left for 15-20 min at room temperature. The jars were then reconnected to the bladder with the gas mixture to test for secondary vacuum, admission of more gas into the jar confirmed the proper functioning of the catalyst. The valves were closed again and the jars disconnected. When no secondary vacuum developed, the catalyst was changed and the procedure repeated. The procedure was repeated three times before the jars were incubated one for 48h and the other for 5 days. The jars used a 'cold' palladium catalyst active at room temperature (Heller, 1954) and

Figure 2.3 Submercible Cell



inactive when wet or in contact with water. The catalyst was rejuvenated by heating at 160°C for 2h (Rosenblatt et al. 1973). Cultures of a strict anaerobe Bacteroides fragilis NCTC 10584 and a strict aerobe Pseudomonas aeruginosa was included in each jar as indicators of anaerobiosis.

For sporulating clostridial counts, 100ml of raw and treated sewage liquors were boiled for 10 min, cooled quickly and then diluted down to 10^{-3}. 10ml aliquots of the neat liquors and of each dilution were filtered in quadruplicate. The filters were layered on to the surface of either neomycin blood agar or perfringens agar and incubated as described above.

After incubation the numbers and types of colonies on each plate were recorded. Several examples of each colonial type were gram-stained and their morphology and staining characteristics recorded. The isolates were inoculated on to fresh plates of the basal blood agar medium in such a manner as to produce individual colonies and incubated aerobically in the presence of 10% CO_2 and anaerobically for 48h.

2.25. Survival Studies

A preliminary study of the survival of coliforms in water was simulated by using a specially constructed submersible cell (Figure 2.3) for in situ survival studies. In the initial study, a water sample was collected from sampling point B (Figure 2.1) and approximately 80ml of this sample was aseptically introduced into the chamber of the sterilized cell. Before submersion of the cell into the stream, a representative water sample in the chamber was withdrawn and transported to the laboratory. Duplicate 0.1ml aliquots of this sample were spread evenly over the surfaces of MacConkey agar

plates and incubated overnight at 37°C for viable counts. At daily intervals for 7 days, representative samples of the contents of the cell *in situ* were taken and 0.1ml of each sample treated in duplicate as stated before.

Once parameters have been established, overnight broth cultures of strains of *E. coli*, Enterobacter species and Klebsiella species were washed twice with ¼ strength Ringer's solution and each strain diluted in Ringer's to give a bacterial density of approximately 2×10^3 organisms ml^{-1}. The diluted strains were introduced into the chamber as before and monitored daily for up to 7 days. The study was carried out separately for each pure strain.

3. RESULTS

TABLE 3.1. Coliforms and Group D Streptococci Densities Above and Below the Sewer Outfall and in the Primary and Humus Settling Tanks (Counts $100ml^{-1}$)

Sample Run No.	Above sewer outfall Coliforms	Above sewer outfall Gp. D. Streptococci	Primary settling tank Coliforms	Humus settling tank Coliforms	Below sewer outfall Coliforms	Below sewer outfall Gp.D. Streptococci
1	2.5×10^3	0.88×10^2			1.7×10^5	0.18×10^4
2	5.1×10^3	0.57×10^2			1.5×10^5	0.17×10^4
3	3.5×10^3	0.52×10^2			4.42×10^5	3.2×10^4
4	3.1×10^3	2.6×10^2			1.5×10^5	1.16×10^5
5	3.25×10^3	3.44×10^2			3.3×10^5	0.87×10^4
6	3.7×10^3	2.0×10^2			1.57×10^5	1.04×10^4
7	4.4×10^3	0.73×10^2	1.36×10^7	8.22×10^5	1.75×10^5	0.47×10^4
8	6.12×10^3	1.1×10^2	5.8×10^7	7.33×10^5	1.42×10^5	0.23×10^4

3. RESULTS

3.1. Enumeration of Bacteria from Faecal Origin

The recorded numbers of viable coliforms and group D streptococci are listed in Table 3.1., according to the sites from which the samples were taken. At the times water samples were collected, the atmospheric condition for each sampling site was quite similar.

Coliforms

3.1.1. Coliform Densities above the Sewer Outfall

The counts of lactose-fermenting coliform bacteria in water samples taken above the sewer outfall ranged from 2.50 - 6.12 x 10^3 $100ml^{-1}$.

3.1.2. Coliform Densities at the Primary and Humus Settling Tanks

On the two occasions when sewage liquor samples were taken from the primary and humus settling tanks, for the enumeration of lactose-positive coliforms, the counts varied between 1.36 - 5.8 x 10^7 $100ml^{-1}$ and 7.33 - 8.22 x 10^5 $100ml^{-1}$ respectively. These samples were taken for the sole purpose of estimating the numbers of faecal *E. coli*.

3.1.3. Coliform Densities Below the Sewer Outfall

On six occasions, below the sewer outfall, the number of lactose-positive coliforms varied between 1.42 - 1.75 x 10^5 $100ml^{-1}$. On two occasions, there was a partial blockage of the sewage treatment system and this elevated the counts to 3.3 and 4.42 x 10^5 $100ml^{-1}$. Thus the system was still relatively efficient.

Group D Streptococci

3.1.4 Group D Streptococci Densities above the Sewer Outfall

The counts for the group D streptococci in samples from above the sewer outfall varied between 0.52 - 3.44 x 10^2 $100ml^{-1}$.

3.1.5. Group D Streptococci Densities Below the Sewer Outfall

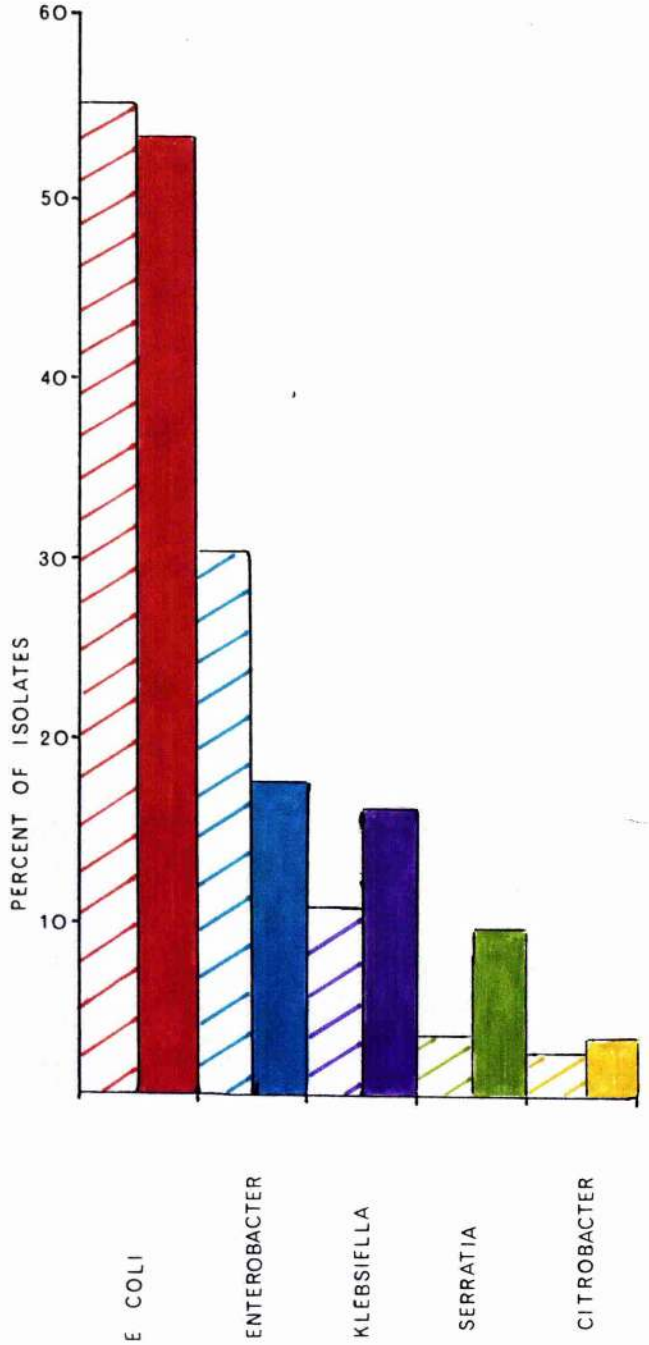
The counts below the sewer outfall varied between $0.17 - 1.04 \times 10^4$ $100ml^{-1}$ on six occasions, and on two occasions when the sewage treatment system was partially blocked the counts rose to 3.2×10^4 and 1.16×10^5 $100ml^{-1}$ respectively. Here again the system was still relatively efficient.

3.2. Identification of the Isolates

3.2.1. Coliforms

On the basis of the results obtained from test procedure 1 outlined in the 'Materials and Methods Section', 55% of the isolates from above the sewer outfall and 53% of the isolates from below the outfall were shown to be strains of E. coli. Those isolates from both sampling points that were not identified as E. coli were then subjected to Test Procedures 2 and 3. Most of these isolates were readily assigned to different groups according to their reactions to those tests. This formed the basis of the presumptive identification of the different genera. The raw data of the biochemical tests employed in the characterization of these presumptively identified coliforms are presented in Appendix 1 (a and b) together with all of the tests carried out during this study, which formed the basis of the Test Procedure 3. At the end of this stage, the coliform isolates presumptively identified belonged to four genera, Citrobacter, Enterobacter, Escherichia and Klebsiella. However, the use of additional diagnostic media, deoxyribonuclease activity (Jeffries et al. 1957), and Tween 80 activity (Lovell and Bibel, 1977) enabled the Serratia species to be differentiated from the others. Two lactose-fermenting isolates from below the sewer outfall could not be presumptively identified. To test the validity of the

FIGURE 3.1 DISTRIBUTION OF COLIFORM GENERA ABOVE AND BELOW THE SEWER OUTFALL.



Key: Crosshatched = Above the outfall.
Blocked = Below the outfall.

identification scheme, up to four examples of each presumptively identified genus and the two isolates for which no presumptive identification was possible, were subjected to identification using the A.P.I. 20E Enterobacteriaceae system (A.P.I. Products Ltd.). The results obtained from this system confirmed the presumptive identification of the genera but no meaningful A.P.I. Profile Number was obtained for either of the two unknown isolates.

3.2.2. Distribution of Coliform Genera from Above and Below the Sewer Outfall

Figure 3.1 shows the per cent distribution of E. coli and other coliform genera isolated from samples taken from above and below the sewer outfall. The percentage of E. coli species in the total coliform population from both sampling points did not vary significantly (55% above; 53% below outfall). One would have expected more E. coli below the sewer outfall than above, since the discharge of domestic sewer effluent into the water course would contribute to an increase in E. coli count at the sampling point below the discharge pipe of the sewer outfall. That Escherichia is the dominant lactose-fermenting coliform genus is not surprising as it is the predominant aerobe in faeces. Other lactose-fermenting members of the coliform group were similarly represented. Whereas Enterobacter species represented 30% of the total coliform population above the sewer outfall only 17% were found below. As far as Klebsiella species were concerned, more were found below (16% than above 10%). Serratia species were also isolated from both sampling points and they constituted 9% and 3% of the total coliform population from below and above the discharge pipe of the sewer outfall respectively. Citrobacter species were present from both sampling points and their numbers were not significantly different. In as much as the identification of the isolates was concerned, the total number of the isolates above the discharge pipe of the sewer outfall presented no difficulty

TABLE 3.2.(a) PERCENTAGES OF COLIFORM ISOLATES OF EACH GENUS FROM ABOVE THE SEWER OUTFALL WITH POSITIVE-REACTION TO TESTS

Test or substrate	Citrobacter spp $_{n = 2}$	Enterobacter spp $_{n = 30}$	Klebsiella spp $_{n = 10}$	Serratia spp $_{n = 3}$
Catalase	100	100	100	100
Gluconate	0	90	100	66
Malonate	100	93	80	66
Urease	0	3	60	0
H_2S	50	0	0	0
Acid from inositol	100	13	80	66
Gas from glucose	100	100	100	100
ONPG	100	100	100	100
MR Test	100	0	0	33
VP Test	0	100	100	100
Indole	0	0	0	0
Gelatin hydrolysis	0	0	0	100
Phenylalanine	0	0	0	0
Citrate as C source	100	100	100	100
Oxidase	0	0	0	0
Motility	100	100	0	100
DNase	0	0	0	100
Tween 80	0	0	0	100

TABLE 3.2.(b) PERCENTAGES OF COLIFORM ISOLATES OF EACH GENUS FROM BELOW THE SEWER OUTFALL WITH POSITIVE-REACTION TO TESTS

Test or substrate	Citrobacter spp $_{n = 3}$	Enterobacter spp $_{n = 17}$	Klebsiella spp $_{n = 16}$	Serratia spp $_{n = 9}$
Catalase	100	100	100	100
Gluconate	0	100	94	89
Malonate	33	76	94	0
Urease	0	6	56	11
H_2S	100	0	0	0
Acid from inositol	33	35	88	100
Gas from glucose	100	100	100	100
ONPG	100	100	100	100
MR Test	100	0	0	0
VP Test	0	100	81	78
Indole	33	0	0	0
Gelatin hydrolysis	0	0	0	100
Phenylalanine	0	0	0	0
Citrate as C source	100	100	100	100
Oxidase	0	0	0	0
Motility	100	100	0	100
DNase	0	0	0	100
Tween 80	0	0	0	100

whereas two below could not be identified.

The percentage isolates of each genus except Escherichia, from each sampling site, with positive results to the various tests performed were calculated (Tables 3.2 (a and b)). It can be seen that not all the isolates of any genus gave 100% positive reaction to all of the tests.

3.2.3. Group D Streptococci (Environmental and Clinical Isolates)

Appendices 2 (a, b and c) show the results of the spectrum of physiological and biochemical tests used to speciate and identify the group D streptococci isolates. All the isolates were gram-positive cocci with varying chain lengths. They failed to release O_2 from H_2O_2. Since the bile-aesculin test was considered paramount in identifying an isolate as a group D streptococcus (Facklam and Moody, 1970; Facklam et al. 1974), all isolates had to give a positive reaction to this test. For an isolate to fit perfectly into the speciation scheme, all the reactions have to agree with those listed in Table 3.3. Even though many aberrant strains or variants within a species were encountered, to best place an isolate into a particular species required the use of the spectrum of reactions. In any case, most of the isolates were placed within a recognised species. On the basis of the scheme in Table 3.3, isolates were speciated into Streptococcus faecalis and its variants liquefaciens and zymogenes, S. faecium, S. faecium var. casseliflavus, S. durans, S. bovis and S. equinus. No Streptococcus avium was isolated and strains that were not identified by this scheme were classified as unidentified group D streptococci. All the control strains (Table 3.4.) were tested alongside with the isolates and they gave the appropriate results. Tables 3.3 (a, b and c) list the percentages of positive reactions of the various species of group D streptococci isolated from the water samples from above and below the discharge pipe of the sewer outfall and the clinical isolates.

TABLE 3.3 (a) PERCENTAGES OF POSITIVE REACTIONS OF GROUP D STREPTOCOCCI SPECIES FROM ABOVE THE SEWER OUTFALL TO THE VARIOUS TESTS

Tests or substrates	S.faecalis var faecalis	S. faecalis var liquefaciens	S. faecalis var zymogenes	S. faecium	S. faecium var casseliflavus	S. durans	S.bovis	S.equinus
β-haemolysis	0	0	100	0	0	100	0	
Group D reaction	100	100	100	100	100	100	100	
Growth at 10 C	100	100	100	100	100	100	0	
Growth at 45 C	100	100	100	100	100	100	100	
Growth at 6.5% NaCl	100	100	100	100	100	100	0	
Growth on 10% BAeM	100	100	100	100	100	100	100	
Growth on 40% BAeM	100	100	100	100	100	100	100	
0.04% Tellurite tolerance	94	100	100	0	100	0	0	
Reduction of 0.1% tetrazolium	75	100	100	7	43	0	0	
Reduction of tetrazolium and decarboxylation of tyrosine (Meads)	100	100	100	7	17	0	0	
Hydrolysis of starch	0	0	0	0	0	0	100	
Liquefaction of gelatin	0	100	0	0	0	0	0	
Pigment production	0	0	0	0	100	0	0	
Apparent pigment production	81	50	100	0	3	0	0	
Acid production from: Mannitol	100	100	100	100	77	0	50	
Glycerol Anaerobic	94	100	100	39	34	0	0	
Arabinose	31	75	0	100	97	17	50	
Melezitose	94	100	100	0	6	0	0	
Sorbitol	100	100	100	46	60	0	0	
Inulin	6	0	0	11	17	0	50	
Melibiose	25	0	0	86	31	50	100	
Lactose	100	100	100	100	100	100	100	
Hydrolysis of arginine	100	100	100	93	100	100	0	
Fermentation of pyruvate	100	100	100	0	0	0	0	
Catalase	0	0	0	0	0	0	0	
Total number tested	16	4	1	28	35	12	2	

TABLE 3. 3(b) PERCENTAGES OF POSITIVE REACTIONS OF GROUP D STREPTOCOCCI SPECIES FROM BELOW THE SEWER OUTFALL TO THE VARIOUS TESTS

Tests or substrates	S.faecalis var. faecalis	S. faecalis var. liquefaciens	S. faecalis var. zymogenes	S. faecium	S. faecium var. casseliflavus	S. durans	S.bovis	S.equinus
β-haemolysis	0	0	100	0	0	100	0	0
Group D reaction	100	100	100	100	100	100	100	100
Growth at 10 C	100	100	100	100	100	100	0	0
Growth at 45 C	100	100	100	100	100	100	100	100
Growth at 6.5% NaCl	100	100	100	100	100	100	0	0
Growth on 10% BAeM	100	100	100	100	100	100	100	100
Growth on 40% BAeM	100	100	100	100	100	100	100	100
0.04% Tellurite tolerance	100	100	100	4	100	0	0	0
Reduction of 0.1% tetrazolium	95	66	100	4	14	0	0	0
Reduction of tetrazolium and decarboxylation of tyrosine (Mead)	97	100	100	0	0	0	0	0
Hydrolysis of starch	0	0	0	0	0	0	50	100
Liquefaction of gelatin	0	100	0	0	0	0	0	0
Pigment production	0	0	0	0	100	0	0	0
Apparent pigment production	87	100	100	0	28	0	0	0
Acid production from: Mannitol	97	100	100	100	100	0	50	0
Glycerol Anaerobic	100	100	100	38	28	0	0	0
Arabinose	8	0	50	100	100	0	100	0
Melezitose	97	100	100	0	0	0	0	0
Sorbitol	95	100	100	4	14	0	0	0
Inulin	0	0	0	0	28	0	100	0
Melibiose	13	0	0	81	57	82	100	100
Lactose	100	100	100	100	100	100	100	0
Hydrolysis of arginine	100	100	100	100	100	100	0	0
Fermentation of pyruvate	100	100	100	0	0	0	0	0
Catalase	0	0	0	0	0	0	0	0
Total number tested	39	3	2	26	7	17	2	1

TABLE 3.3(c) PERCENTAGES OF POSITIVE REACTIONS OF GROUP D STREPTOCOCCI SPECIES FROM CLINICAL SOURCES TO THE VARIOUS TESTS

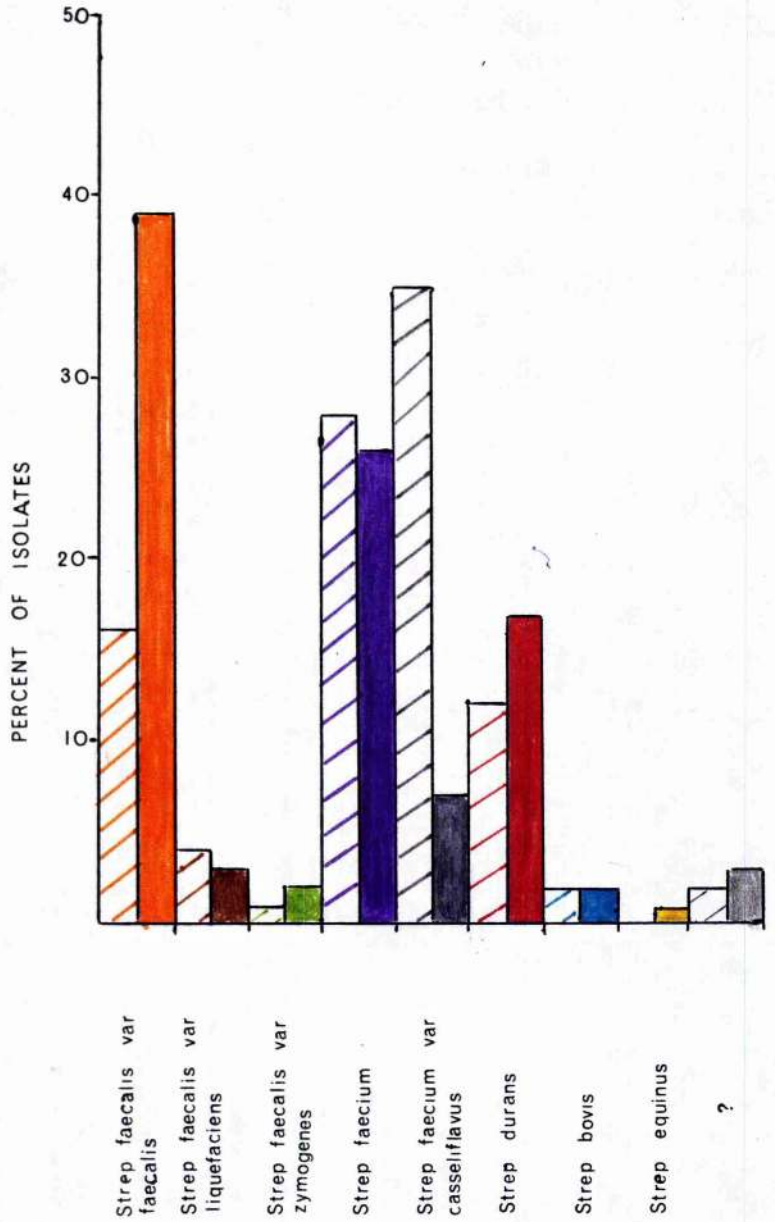
Tests or substrates	S.faecalis var. faecalis	S. faecalis var liquefaciens	S. faecalis var zymogenes	S. faecium	S. faecium var casseliflavus	S. durans	S.bovis	S.equinus
β-haemolysis	0	0	100	0	0	100		
Group D reaction	100	100	100	100	100	100		
Growth at 10 C	100	100	100	100	100	100		
Growth at 45 C	100	100	100	100	100	100		
Growth at 6.5% NaCl	100	100	100	100	100	100		
Growth on 10% BAeM	100	100	100	100	100	100		
Growth on 40% BAeM	100	100	100	100	100	100		
0.04% Tellurite tolerance	100	100	100	0	100	0		
Reduction of 0.1% tetrazolium	94	100	100	0	100	0		
Reduction of tetrazolium and decarboxylation of tyrosine (Meads)	95	100	100	0	0	0		
Hydrolysis of starch	0	0	0	0	0	0		
Liquefaction of gelatin	0	100	0	0	0	0		
Pigment production	0	0	0	0	100	0		
Apparent pigment production	89	93	100	0	100	0		
Acid production from: Mannitol	97	100	100	100	100	0		
Glycerol Anaerobic	92	100	100	0	0	0		
Arabinose	0	0	0	50	100	0		
Melezitose	83	100	87	0	0	0		
Sorbitol	98	100	93	0	0	0		
Inulin	0	0	0	0	100	0		
Melibiose	0	0	0	100	100	0		
Lactose	100	100	100	100	100	100		
Hydrolysis of arginine	100	100	100	100	100	100		
Fermentation of pyruvate	100	100	100	0	0	0		
Catalase	0	0	0	0	0	0		
Total number tested	64	14	15	2	1	3		

TABLE 3.4 LIST OF CONTROL GROUP D STREPTOCOCCI STRAINS

STRAIN No.	Species
CCM 2479	*S. faecium* var. *casseliflavus*
NCDC 596	*S. durans*
NCDC 1037	*S. equinus*
NCTC 7171	*S. faecium*
NCTC 8177	*S. bovis*
NCTC 9938	*S. avium*
M 775	*S. faecalis* var. *faecalis*
NCTC 8131	*S. faecalis* var. *liquefaciens*
NCTC 5975	*S. faecalis* var. *zymogenes*

S. faecalis and its variants liquefaciens and zymogenes were nearly always biochemically typical, and were differentiated by the haemolytic activity of the zymogenes variant and gelatinase production of the liquefaciens variant. S. faecalis var. faecalis was not β-haemolytic and did not liquefy gelatin. Besides the usual reaction on 10% and 40% bile-aesculin agars, growth at 10°C and 45°C, growth in 6.5% NaCl., the majority of the S. faecalis isolates produced acid from mannitol, melezitose and lactose. In addition they hydrolysed arginine and fermented pyruvate (Gross et al. 1975). The pyruvate and arginine tests gave unequivocal results and are, therefore, excellent key tests. The concurrent use of these two tests along with the others listed in Table 3.3 gave reliable results for the identification of S. faecalis and its variants. S. faecium isolates were, on the other hand, nearly always melibiose positive and always fermented arabinose and hydrolysed arginine. In addition, they were always lactose and mannitol-fermenting. For an isolate to be placed in the S. faecium var. casseliflavus group, it had to produce yellow pigment on blood and sucrose agar plates and also have had the following characteristics - resistance to potassium tellurite with the production of grey colonies on the agar medium and fermentation of lactose. S. faecium var. casseliflavus strains had to have at least one of the following properties, fermentation of mannitol, sorbitol, arabinose and hydrolysis of arginine. This relatively poorly defined variant of S. faecium, appeared to share common characteristics with S. faecalis and S. faecium. The positive reaction with arabinose showed its relationship with S. faecium and the positive reaction on potassium tellurite its relationship with S. faecalis.

FIGURE 3.2 SPECIES DISTRIBUTION OF GROUP D STREPTOCOCCI ABOVE AND BELOW THE SEWER OUTFALL.



Key: Crosshatched - Above the outfall.
Blocked - Below the outfall.

S. durans was recognised by its lack of reducing power and fermentative ability. To place an isolate in this group the organism had to have a β-haemolytic activity on blood agar and ferment lactose. The S. durans species isolated characteristically failed to ferment mannitol but did or did not ferment melibiose or arabinose.

S. bovis were unable to initiate growth at 10°C unable to grow in a medium containing 6.5% (w/v) NaCl, and unable to ferment pyruvate or hydrolyse arginine. But most hydrolysed starch and all isolates fermented melibiose.

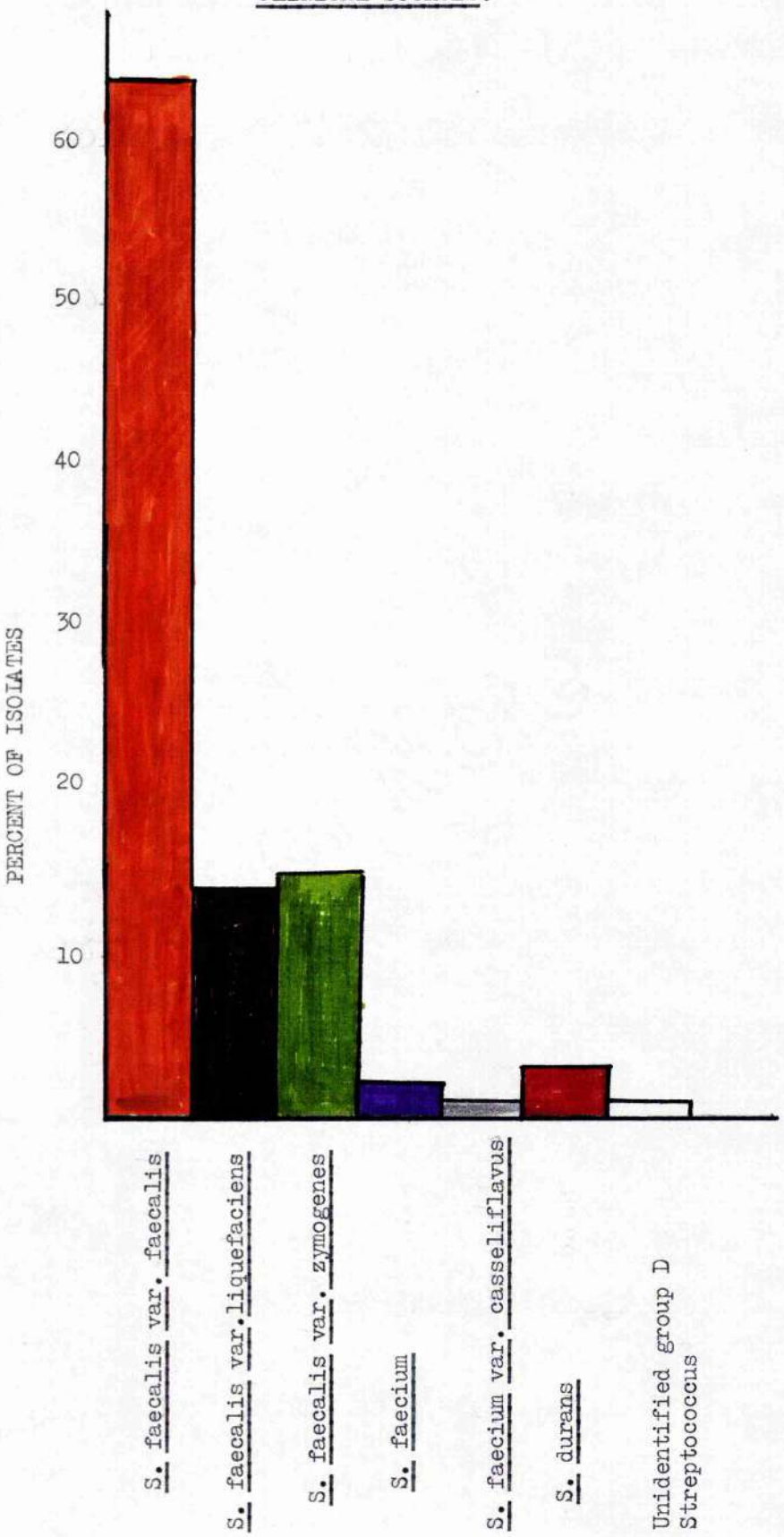
The only S. equinus species, like the S. bovis isolates, was unable to initiate growth at 10°C, or in 6.5% (w/v) NaCl. In addition, it failed to ferment pyruvate and was unable to hydrolyse arginine but was able to hydrolyse starch.

3.2.4. Species Distribution of Group D Streptococci Isolates from Above and Below the Sewer Outfall

The results of the speciation of the group D streptococci from both sampling points is shown in Figure 3.2. From above the sewage plant, 35% of the isolates were identified as S. faecium var. casseliflavus and represented the dominant species. The other species (or variants of a species) identified in order of prevalence were S. faecium (28%), S. faecalis var. faecalis (16%), S. durans (12%), S. faecalis var. liquefaciens (4%), S. bovis (2%) and S. faecalis var. zymogenes (1%). Two of the isolates were unidentified.

Speciation of the isolates from below the outfall revealed a different picture. Here, S. faecalis var. faecalis represented the dominant species and constituted 39% of the total isolates. Again, the species (or variants of a species) identified in order of prevalence were S. faecium (26%), S. durans (17%), S. faecium var. casseliflavus (7%), S. faecalis var. liquefaciens (3%), S. faecalis var. zymogenes (2%), S. bovis (2%), S. equinus (1%) while the remaining 3% were unidentified.

FIGURE 3.3. SPECIES DISTRIBUTION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES.



3.2.5. Species Distribution of Group D Streptococci from Clinical Sources

One hundred presumptive group D streptococcal strains of diverse clinical origin which were obtained from Victoria Hospital, Kirkcaldy, were speciated along with the environmental isolates. The distribution of the species (or variants of a species) is shown in Figure 3.3. It can be seen that *S. faecalis var. faecalis* is the dominant species constituting 64% of the total isolates. The other two major strains identified were the variants of *S. faecalis*. *S. faecalis var. zymogenes* constituted 15% of these whilst 14% were *S. faecalis var. liquefaciens*. *S. durans* species constituted 3%, *S. faecium* 2% and *S. faecium var. casseliflavus* 1%. One isolate was unidentified. None of the isolates was identified as *S. bovis* or *S. equinus*.

3.3. Coagglutination Grouping

All the isolates reacted with the coagglutination grouping method.

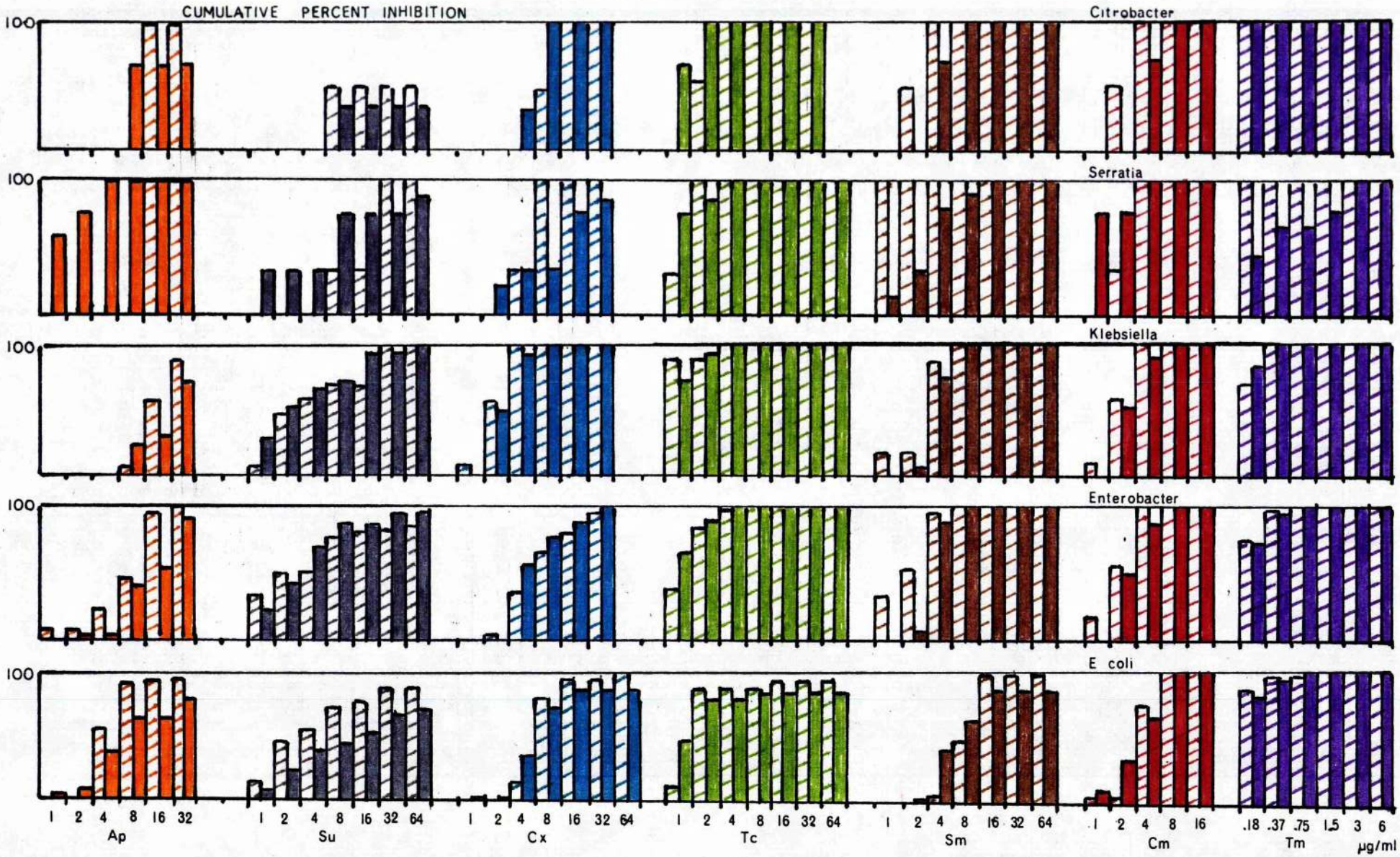
3.4. Faecal Coliforms (FC): Faecal Streptococci (FS) Ratios

Using the FC and FS numbers from each of the sampling runs, the FC:FS ratio was calculated (Table 3.5). The FC counts in samples above the outfall ranged from 1375 - 3366 organisms $100ml^{-1}$. The FS counts on the other hand, ranged from 52 - 344 organisms $100ml^{-1}$. Below the outfall, however, the FC counts ranged from 74,200 - 234 260 organisms $100ml^{-1}$, whereasthe FS count ranged from 1,700 - 160,000 organisms $100ml^{-1}$. All of the FC:FS ratios except one, were greater than 4 indicating that the pollution was predominantly from human sources. On the two occasions when there was a partial blockage of the plant the FC:FS ratio, curiously enough, did not differ significantly from those calculated on the other occasions.

TABLE 3.5. Faecal Coliform (FC). Faecal Streptococci (FS) and FC/FS Ratios on Water Samples Taken from Above and Below the outfall

	Above the outfall			Below the outfall		
Sampling Run	FC $100ml^{-1}$	FS $100ml^{-1}$	FC/FS	FC $100ml^{-1}$	FS $100ml^{-1}$	FC/FS
1	1375	88	15.62	90,100	1,800	50.00
2	2805	57	49.21	79,500	1,700	46.76
3	1925	52	37.00	234,260	32,000	7.32
4	1705	260	6.55	79,500	160,000	0.49
5	1787	344	5.19	174,900	8,700	20.00
6	2035	200	10.17	83,210	10,400	8.00
7	2420	73	33.15	92,750	4,700	19.73
8	3366	110	30.6	74,200	2,300	32.26

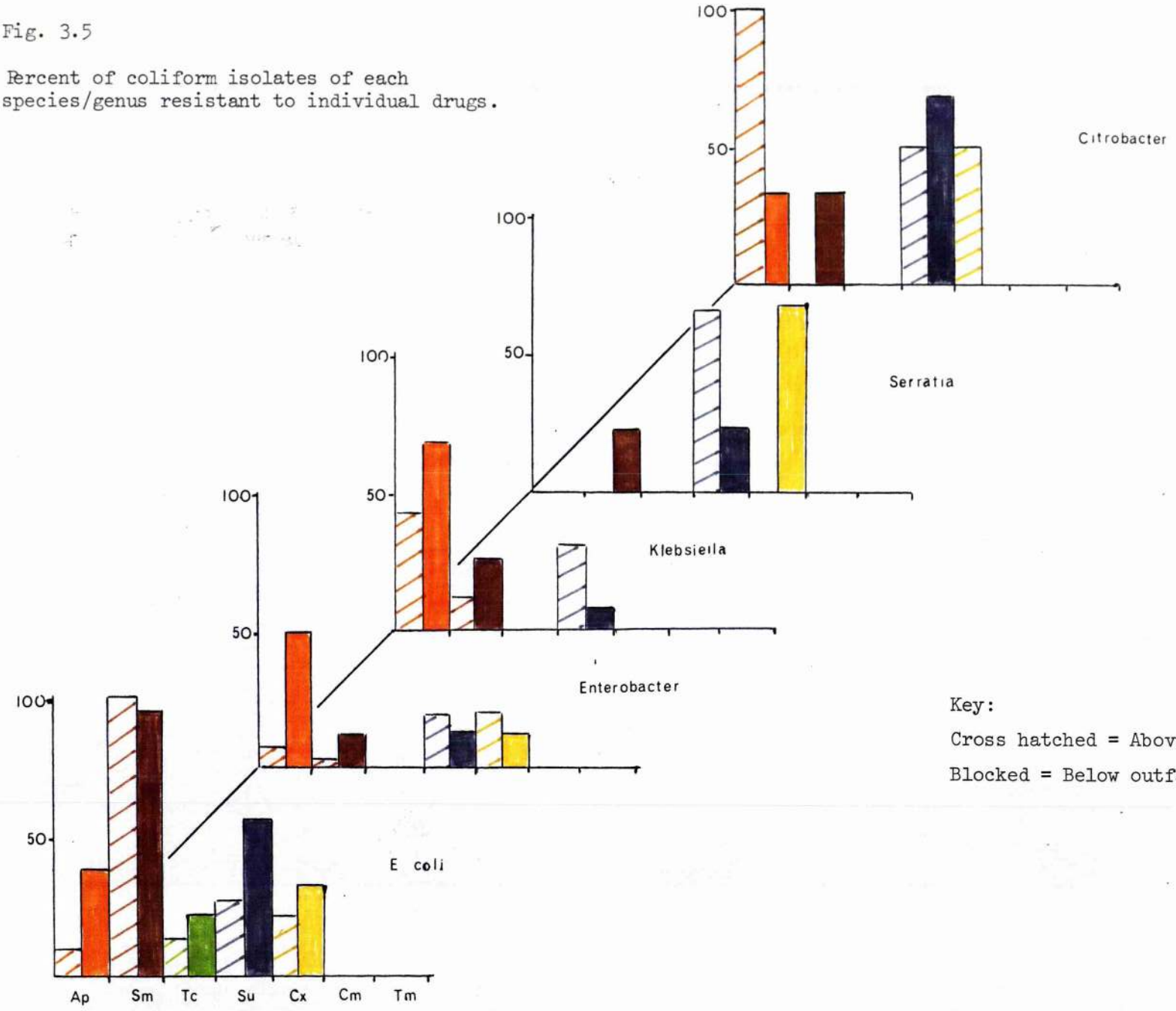
FIGURE 3.4 SUSCEPTIBILITY OF COLIFORMS TO 7 ANTIBACTERIAL AGENTS.



Key: Crosshatched = Above outfall
Blocked = Below outfall

Fig. 3.5

Percent of coliform isolates of each species/genus resistant to individual drugs.



Key:

Cross hatched = Above outfall

Blocked = Below outfall

3.5. Drug Susceptibility Tests

3.5.1. Coliform Isolates from Above and Below the Sewer Outfall

Appendices 3 (a and b) show the results of the M.I.C. determinations for all the coliform isolates. The results from these were used in the preparation of Figure 3.4 as the cumulative percentage of each species or genus inhibited at a given M.I.C. ($\mu g.ml^{-1}$) levels for the seven antimicrobial agents investigated. Resistance was observed to 5(Ap, Sm, Tc, Cx, Su) of the 7 drugs used. As far as resistance to these drugs was concerned, the resistant strains of each species or genus exhibited varying levels of resistance.

From the data in Figure 3.4, the percentage of isolates of each species or genus, from above and below the sewer outfall, which displayed resistance to the individual drugs was determined. The results of this determination are presented in Table 3.6 and in Figure 3.5. It was found that (i) resistance was more common amongst E. coli isolates than any other genera and (ii) resistance to Ap or Su was more common than to the other drugs, with the exception of resistance to Sm in the E. coli isolates.

3.5.2. Statistical Significance of Resistance Amongst Coliforms

The results of the statistical analyses are shown in Table 3.6 together with the degree of significance, where appropriate. Statistical analyses of the results with the five drugs to which any resistance was shown, revealed that there was a statistical significant increase in resistance to Ap and Su in E. coli isolates from below than above; and in Ap resistance in enterobacter species from below than above. In addition there was a statistically significant increase in resistance among isolates taken from below the sewer outfall (x^2 = 12.43 which is significant at the 0.5% level).

TABLE 3.6 ISOLATES OF EACH COLIFORM GENUS RESISTANT TO THE ANTIMICROBIAL AGENTS INVESTIGATED

	Nr. of isolates	Ap Nr.(%) resistant	Ap S/N.S.	Sm Nr.(%) resistant	Sm S/N.S.	Tc Nr.(%) resistant	Tc S/N.S.	Su Nr.(%) resistant	Su S/N.S.	Cx Nr.(%) resistant	Cx S/N.S.	Cm/Tm
E. coli (above)	55	5(9.09)	S	55(100.00)	N.S.	6(10.91)	N.S.	15(27.28)	S	11(20.00)	N.S.	No resistant isolates found.
E. coli (below)	53	21(36.62)	(0.5%)	51(96.23)		9(16.98)		30(56.61)	(0.5%)	17(32.10)		
Enterobacter spp. (above)	30	2(6.67)	S	1(3.33)	N.S.	0(-)	-	6(20.00)	N.S.	6(20.00)	N.S.	
Enterobacter spp. (below)	17	8(47.06)	(0.2%)	2(11.77)		0(-)		2(11.77)		2(11.77)		
Klebsiella spp. (above)	10	4(40.00)	N.S.	1(10.00)	N.S.	0(-)		3(30.00)	N.S.	0(-)	-	
Klebsiella spp. (below)	16	11(68.75)		4(25.00)		0(-)		1(6.25)		0(-)		
Serratia spp. (above)	3	0(-)	-	0(-)	N.S.	0(-)	-	2(66.67)	N.S.	0(-)	N.S.	
Serratia spp. (below)	9	0(-)		2(22.23)		0(-)		2(22.22)		2(22.22)		
Citrobacter spp. (above)	2	2(100.00)	-	0(-)	-	0(-)	-	1(50.00)	N.S.	1(50.00)	N.S.	
Citrobacter spp. (below)	3	1(33.33)		1(33.33)		0(-)		2(66.67)		0(-)		

(above) : Isolates from above the sewer outfall.

(below) : Isolates from below the sewer outfall.

S./N.S.: Significant/Not significant statistical difference in resistance at least at the 0.5% level between the two sets of isolates.

TABLE 3.7 EVIDENCE OF MULTIPLE RESISTANCE IN COLIFORMS

Number of drugs to which resistance is shown	No. of strains	Number of strains (and percentages) showing resistance to varying numbers of drugs tested. 1	%	2	%	3	%	4	%	5	%
E. coli (above)	55	34	62	12	22	5	9	1	2	3	5
E. coli (below)	53	11	21	15	28	17	32	9	17	0	-
Enterobacter spp. (above)	30	8	27	2	7	1	3	0	-	0	-
Enterobacter spp. (below)	17	7	41	2	12	1	6	0	-	0	-
Klebsiella spp. (above)	10	2	20	3	30	0	-	0	-	0	-
Klebsiella spp. (below)	16	8	50	4	25	0	-	0	-	0	-
Serratia spp. (above)	3	2	67	0	-	0	-	0	-	0	-
Serratia spp. (below)	9	1	11	1	11	1	11	0	-	0	-
Citrobacter spp. (above)	2	0	-	2	100	0	-	0	-	0	-
Citrobacter spp. (below)	3	1	33	0	-	1	33	0	-	0	-

Percentages have been given to the nearest whole number.

3.5.3. Evidence for Multiple Drug Resistance in the Coliform Isolates

The number and per cent of each species or genus isolated from above and below the sewer outfall showing resistance to one or more of the drugs tested is given in Table 3.7. *E. coli* isolates by far outnumber any of the other genera in the display of multiple resistance. Multiple resistance as used in this context refers to the simultaneous display of resistance by any one isolate, to three or more of the drugs tested. Whereas *E. coli* isolates from both sampling points displayed resistance to up to four drugs, resistance to five drugs was displayed by three isolates from above the sewer outfall. Very few of the other genera were resistant to more than three of the drugs tested. Simultaneous resistance to three drugs was found in Enterobacter, Citrobacter and Serratia isolates but the numbers of isolates of the latter two genera were too low to make any conclusions.

3.5.4. Resistance Patterns

Table 3.8 shows the distribution of the various resistance patterns amongst the multiple-resistant isolates together with the site from which they were isolated. A total of nine resistance patterns were exhibited by 39 multiple-resistant organisms. The most common resistance pattern was SmSuCx (11 isolates) followed by ApSmSuCx (8 isolates) and SmTcSu (7 isolates). Three *E. coli* isolates, all from above the sewer outfall, displayed a pattern of ApSmTcSuCx. 29 isolates (74.36%), from below the sewer outfall, were multiple-resistant as compared with 10 isolates (25.36%) from above. 90% of the multiple-resistant isolates from above the sewer outfall and approximately the same percentage from below, were *E. coli*.

TABLE 3.8 DISTRIBUTION OF ANTIBIOTIC RESISTANCE PATTERNS AMONGST MULTIPLE-RESISTANT COLIFORM ISOLATES FROM ABOVE AND BELOW THE SEWER OUTFALL

				Source or Origin			
	Antibiotic resistance pattern	Isolates No.	%	Above sewer outfall Species/Genus	No.	Below sewer outfall Species/Genus	No.
	Ap Sm Cx	1	2.56			E. coli	1
	Ap Su Cx	1	2.56	Enterobacter	1		
	Ap Sm Su	5	12.82			E. coli Citrobacter	4 1
	Sm Tc Cx	1	2.56	E. coli	1		
	Sm Su Cx	11	28.20	E. coli	4	E. coli Enterobacter Serratia	5 1 1
	Sm Tc Su	7	17.95			E. coli	7
	Ap Sm Su Cx	8	20.51	E. coli	1	E. coli	7
	Sm Tc Su Cx	2	5.13			E. coli	2
	Ap Sm Tc Su Cx	3	7.69	E. coli	3		
Total	9	39			10		29

Fig. 3.6. Susceptibility of group D streptococci from clinical sources 6 antibacterial agents.

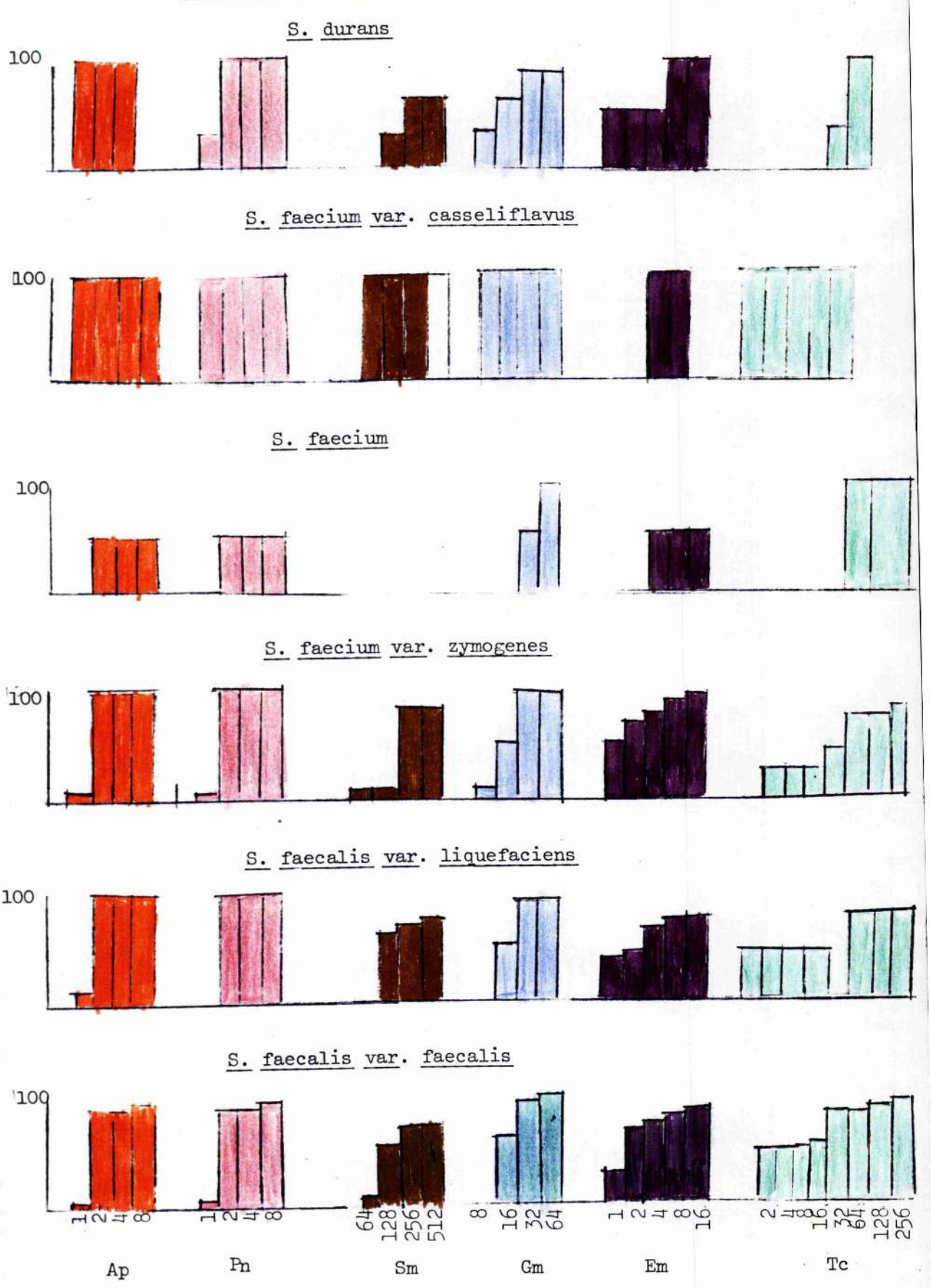
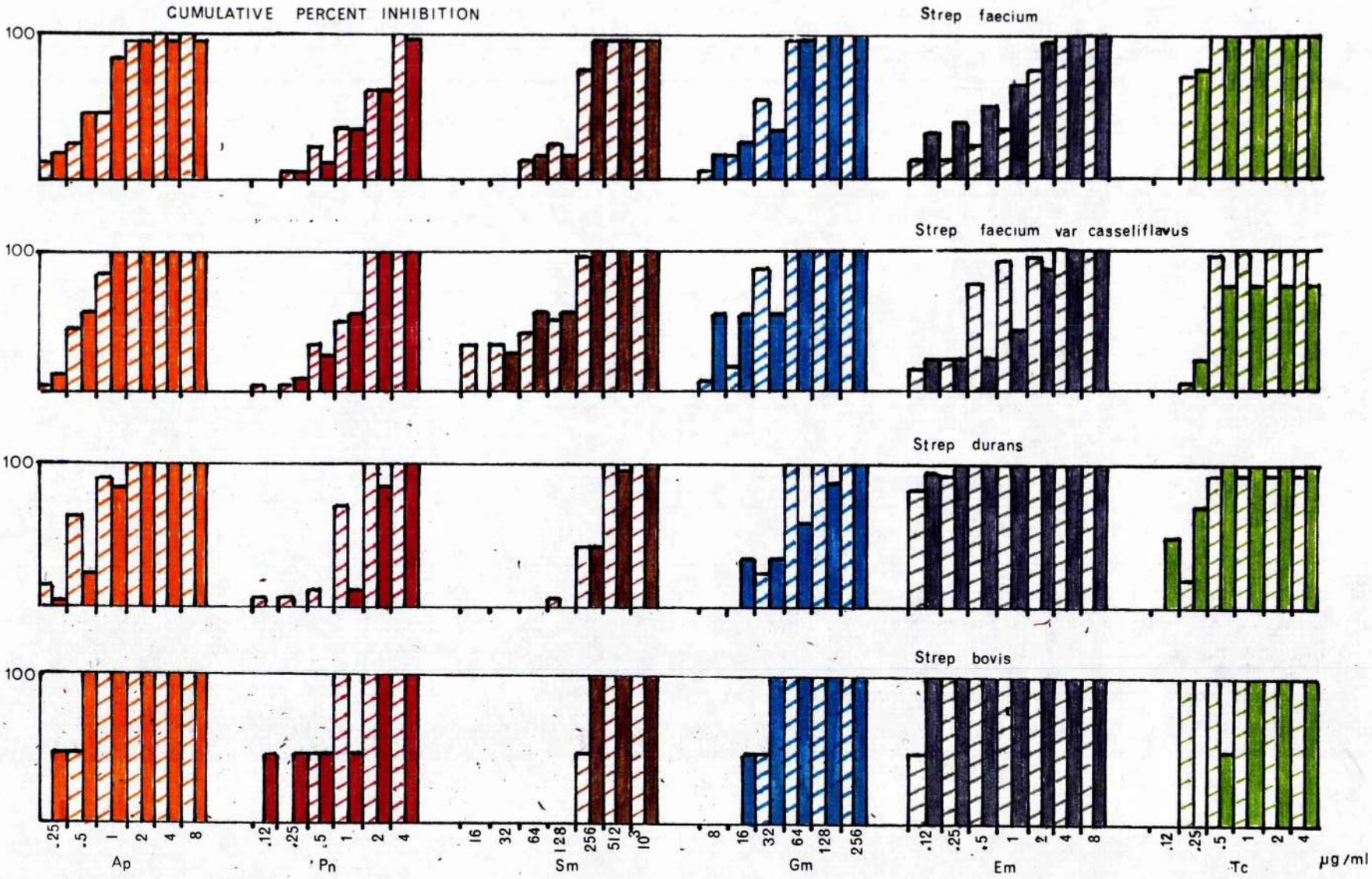
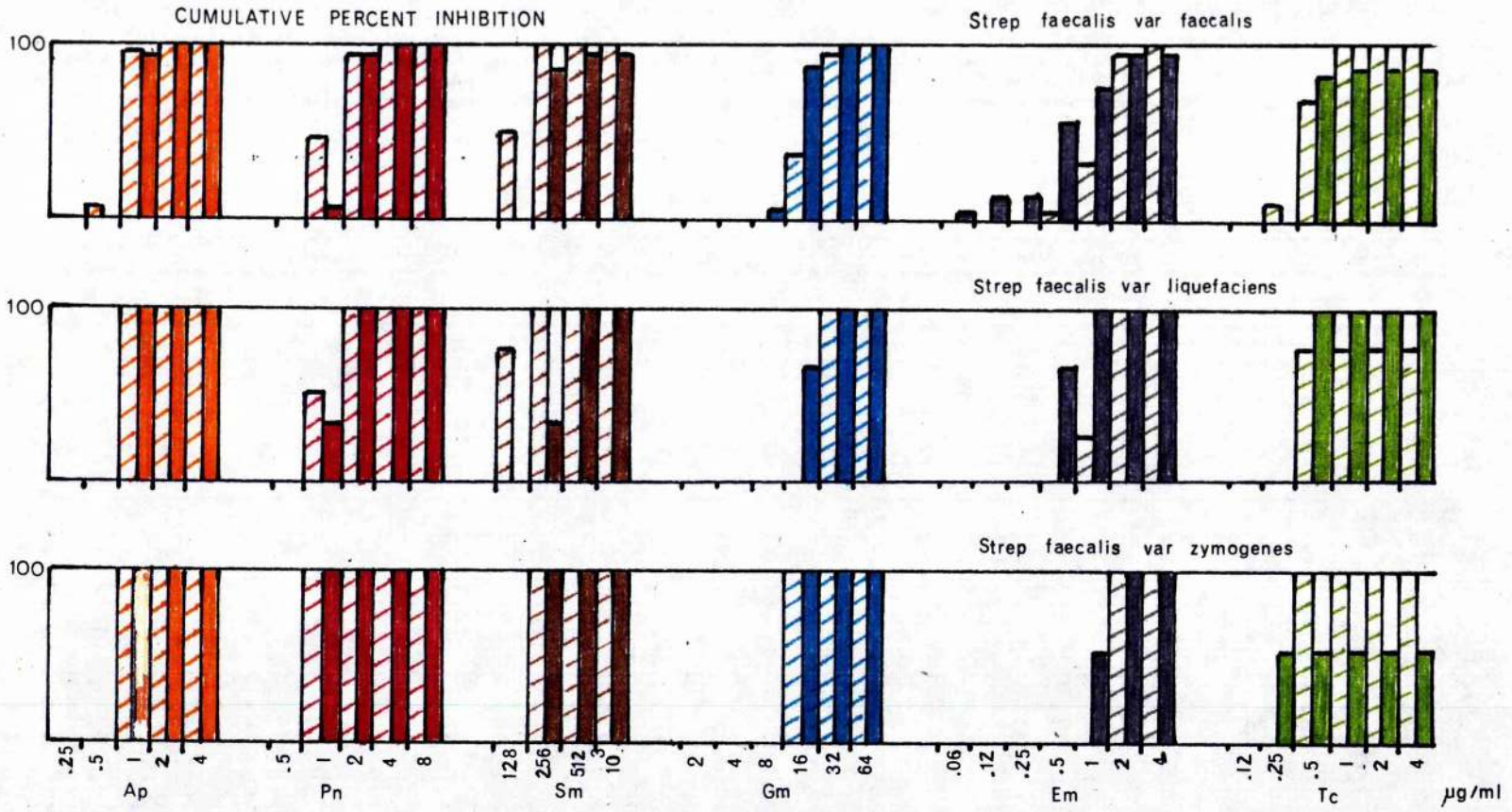


FIGURE 3.6 (Contd.) SUSCEPTIBILITY OF GROUP D STREPTOCOCCI TO 6 ANTIBACTERIAL AGENTS



Key: Cross hatched = Above outfall : Blocked = Below outfall.

FIGURE 3.6. SUSCEPTIBILITY OF GROUP D STREPTOCOCCI TO 6 ANTIBACTERIAL AGENTS.



Key: Cross hatched = Above outfall

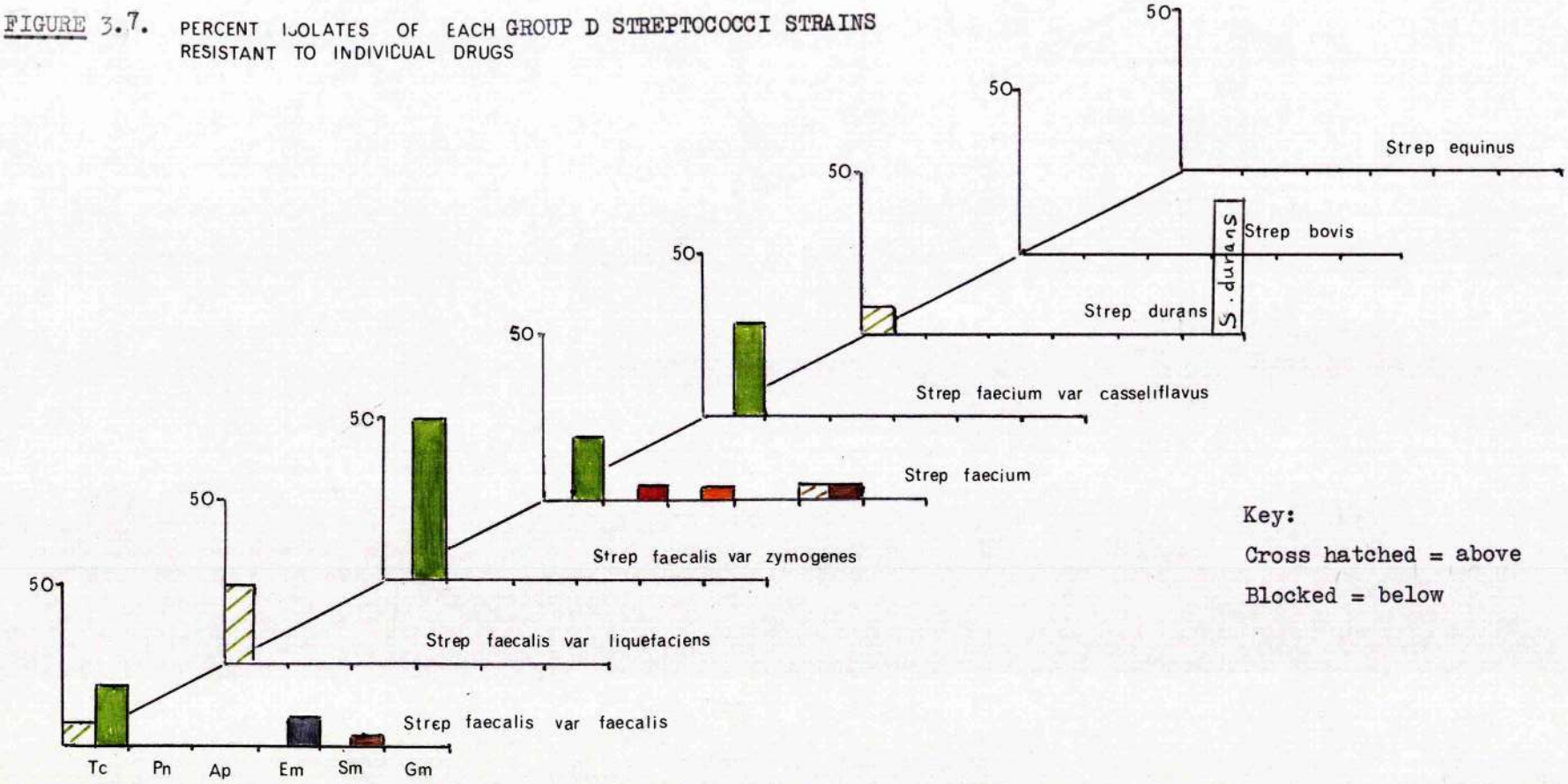
Blocked = Below outfall

3.5.5. Drug Resistance Among Group D Streptococci

Appendices 4 (a,b and c) show the results of the M.I.C. determinations on the group D streptococci isolates from above and below the sewer outfall and on the group D clinical isolates. The results shown in the appendices were used in the preparation of Figure 3.6 which gives the cumulative percentage of each species (or variant of a species) inhibited at a given M.I.C. ($\mu g.ml^{-1}$) levels for the six drugs tested. Resistance to Ap, Pn, Sm, Gm and Tc was seen but Tc was the only drug to which any appreciable number of strains displayed resistance. As far as resistance to Em was concerned, none of the isolates from above the sewer outfall, exhibited any resistance to this drug. However, a few of the clinical isolates and even fewer isolates from below the sewer outfall did display resistance to Em. Although Gm and Sm-resistant strains were encountered, strains resistant to Gm were characteristically from below the sewer outfall, and were not simultaneously reistant to Sm. Strains resistant to Sm were encountered from both environmental and clinical sources, but the significance of streptococcal resistance to this drug is doubtful, since these organisms are known to be intrinsically Sm-resistant (Phillips, 1975). Where there was resistance to Ap (as found in two isolates), simultaneous resistance to Pn was also displayed.

From the data in Figure 3.6 the percentage of isolates of each strain from above and below the sewer outfall, resistant to individual drugs was determined. The result of this is shown in Figure 3.7. Whereas *S. bovis* and *S. equinus* species were sensitive to all of the drugs *S. faecium* species displayed the widest range of resistance.

FIGURE 3.7. PERCENT ISOLATES OF EACH GROUP D STREPTOCOCCI STRAINS RESISTANT TO INDIVIDUAL DRUGS



Key:

Cross hatched = above

Blocked = below

Statistical analysis showed no significant increase in resistance between isolates from above and below the sewer outfall.
As far as resistance amongst the clinical isolates was concerned, 55% of the isolates displayed resistance to Tc. None of the isolates were resitant to Gm. Of the isolates (6%) resistant to Em, the level of resistance in all the cases was very high. There was no Ap or Pn resistant strains except one which was simultaneously resistant to both of the drugs. The percentage isolates resistant to individual drugs is shown in Figure 3.7.

3.5.6. Evidence for Multiple Drug Resistance in Group D Streptococci

As far as the incidence of multiple drug resistance amongst the group D streptococci was concerned, evidence for the occurrence of this phenomenon was not as frequent as amongst the coliforms. Although 7 strains exhibited multiple drug resistance, their distribution was restricted to two sources (i) 2 strains were from below the sewer outfall and (ii) 5 strains were of clinical origin. The predominant group D streptococci displaying multiple drug resistance were S. faecalis and its variants liquefaciens and zymogenes.

With regard to the resistance patterns, Sm Em Tc, Ap Pn Sm Tc, or Ap Pn Sm Em Tc were the three permutations in evidence. The Sm Em Tc resistance pattern was confined to strains of S. faecalis and its variants whereas the other two patterns were displayed by two individual strains of S. faecium.

3.6. Possible Evidence for Plasmid Mediated Drug Resistance - Demonstration of transferability by conjugation.

3.6.1. Transfer of Drug Resistance Factors from Coliforms (donors) to E. coli K12 (recipient).

The incidence and transferability of drug resistance factors from multiple drug resistant coliforms to E. coli K-12 is shown in Table 3.9 as the number and percentage transfer of single drugs or drugs added in combination. It can be seen that all the coliform isolates transferred two or more of their resistance markers. In most cases where single drugs were involved, the markers were expressed within 24h whereas in all cases where drugs were added in combination evidence for transfer did not become apparent until 72h. With regard

TABLE 3.9

QUALITATIVE DRUG RESISTANCE TRANSFER FROM COLIFORMS TO E. COLI K-12

Resistance pattern	No.of isolates	Growth on Nal + single drug after:			% transfer	Growth on Nal + combination of drugs after:					% transfer
		24h	48h	72h		24h	48h	72h	96h	120h	
ApSmCx	1	+	+	+	100	-	-	-	-	-	0
ApSuCx	1	-	-	+	100	-	-	+	+	+	100
ApSmSu	5	+	+	+	100	-	-	+(1)	+	+	20
SmTcCx	1	+	+	+	100	-	-	-	-	-	0
SmSuCx	11	-	-	+(2)	54.54	-	-	-	-	-	0
SmTcSu	7	-	-	+	100	-	-	-	-	-	0
ApSmSuCx	8	+	+	+	100	-	-	+	+(3)	+	12.5
SmTcSuCx	2	+	+	+	100	-	-	-	-	-	0
ApSmTcSuCx	3	+	+	+	100	-	-	-	-	-	0

\+ = growth
\- = no growth

(1) 1 out of 5 isolates
(2) 6 out of 11 isolates
(3) 1 out of 8 isolates

Transconjugants were selected on a set of nalidixic acid plates, the plates in the set, in addition to Nal contained one of the drugs to which the donor was resistant., i.e. each transconjugant was selected against Nal + each of the drugs to which the donor was resistant.

to the transferability of single drugs, 5 isolates out of 11 possessing the resistance markers Sm Su Cx could not transfer Cx to the E. coli recipient. As far as the detection of transfer in cases where drugs had been added in combination was concerned, transferability was detected in 3 out of the 9 resistance pattern groups. The only isolate (Enterobacter spp.), with a pattern Ap Su Cx, was able to express each marker on a combination plate. There were two other groups where transconjugants grew on combination plates. Within one group (Ap Sm Su), which consisted of 4 E. coli and 1 Citrobacter species, such growth on combination plates was detected with one of the 4 E. coli strains. Wihtin the second group (Ap Sm Su Cx), which consisted of 8 E. coli strains, such growth was only detected with one of these strains as donor.

Table 3.10 shows the results of the quantitative study of the efficiency of drug resistance transfer to E. coli K-12 from various coliforms. It was observed that when both the donor and recipient strains were E. coli, the efficiency of transfer was significantly greater than that from other coliforms other than E. coli, to E. coli K-12. To investigate this differential efficiency of transfer between different genera, a study was initiated, the results of which are presented in Table 3.11.

For this study, Ap resistance transfer was used as the marker. The transfer frequencies were calculated after 4h of mating and varied between 0.91×10^{-5} - 0.6×10^{-3} recipient cell^{-1}. The frequency of transfer was highest (0.6×10^{-3}) when both donor and recipient strains were E. coli, and lowest (0.91×10^{-5}) when the recipient was a Serratia species. Citrobacter species ranked next to E. coli, followed by Enterobacter species and Klebsiella species. An attempt was then made to develop a postulate to explain this differential in efficiency.

TABLE 3.10 QUANTITATIVE DRUG RESISTANCE TRANSFER FROM COLIFORMS TO E. COLI K-12

Donor Strain	Resistance markers	Markers transferred	Transfer frequency
CB 18 *E. coli*	Ap Sm Cx	Ap	0.61×10^{-3}
CA 22 *Enterobacter spp.*	Ap Su Cx	Ap	0.22×10^{-5}
CB 1 *Citrobacter spp.*	Ap Sm Su	Ap	0.45×10^{-4}
CB 80 *E. coli*	Ap Sm Su	Ap	0.19×10^{-3}
CA 54 *E. coli*	Sm Tc Cx	Tc	0.30×10^{-2}
CB 9 *E. coli*	Sm Su Cx	Su	0.45×10^{-3}
CB 62 *Serratia spp.*	Sm Su Cx	Su	0.32×10^{-5}
CB 81 *Enterobacter spp.*	Sm Su Cx	Sm	0.60×10^{-5}
CB 74 *E. coli*	Sm Tc Su	Tc	0.63×10^{-3}
CB 28 *E. coli*	Ap Sm Su Cx	Ap	0.37×10^{-3}
CA 67 *E. coli*	Sm Tc Su Cx	Tc	0.42×10^{-3}
CA 93 *E. coli*	Ap Sm Tc Su Cx	Ap	0.14×10^{-3}

TABLE 3.11 EFFICIENCES OF AMPICILLIN TRANSFER FROM A MULTIPLE-RESISTANT E. COLI ISOLATE (DONOR) TO NALIDIXIC ACID-RESISTANT MUTANTS OF OTHER COLIFORM GENERA AS RECIPIENTS.

Donor Strain	Recipient strain	Efficiency of transfer
E. coli	*E. coli*	0.6×10^{-3}
E. coli	*Citrobacter spp.*	0.51×10^{-4}
E. coli	*Enterobacter spp.*	0.38×10^{-4}
E. coli	*Klebsiella spp.*	0.02×10^{-4}
E. coli	*Serratia spp.*	0.91×10^{-5}

3.6.2. Relative Efficiency of Transfer

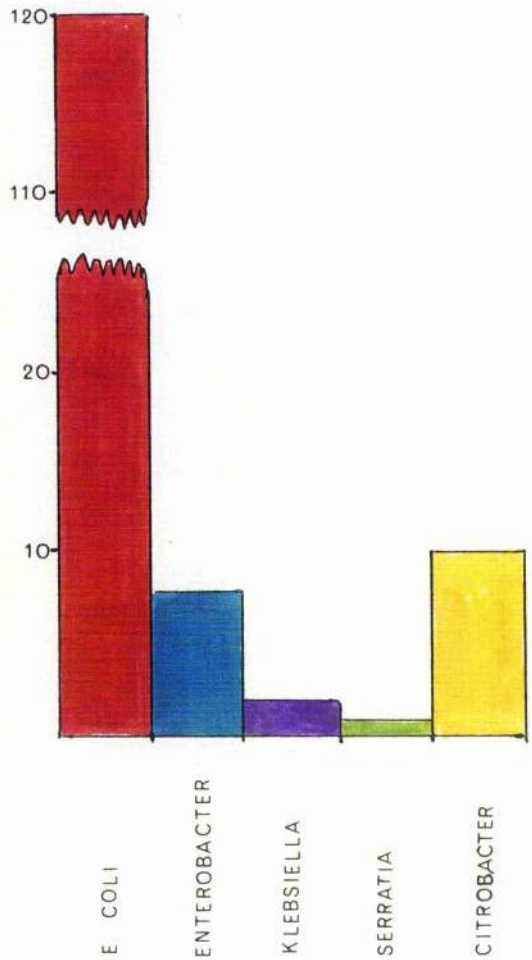
Figure 3.8 shows the relative efficiency of transfer from an *E. coli* donor to Nal^r mutants of *E. coli*, and to similar mutants of other coliform genera used as recipients, in addition to their G + C ratios, as quoted by Bergey (1974) and by Wilson and Miles (1975). The results obtained from Table 3.11 were also used in the determination of the relative efficiencies of transfer. To find the relative efficiency of transfer for each genus, the efficiency of drug transfer from *E. coli* (donor) to Serratia species (recipient) was assumed to be 1, and using this figure, the efficiency for each of the other genera was calculated. As a result of this conversion, the following relative transfer efficiencies were obtained:

Donor	Recipient	Relative transfer efficiency
E. coli	*E. coli*	120
E. coli	*Citrobacter spp.*	10
E. coli	*Enterobacter spp.*	8
E. coli	*Klebsiella spp.*	2
E. coli	*Serratia spp.*	1

If one takes any overlap in G + C ratios between genera to infer a genetic relationship, it could be postulated that this is one possible explanation for the differences in the inter-generic relative transfer efficiencies which have been determined. The inter-generic transfer efficiency between *E. coli* and Klebsiella species, may in addition be depressed since Klebsiella species are non-motile which would reduce the probability of effective pair formation during conjugation. Since the efficiency of resistance transfer between *E. coli* species is so much greater than that for inter-generic transfer, this could also explain why resistance and particularly multiple drug resistance is predominant among the *E. coli* isolates.

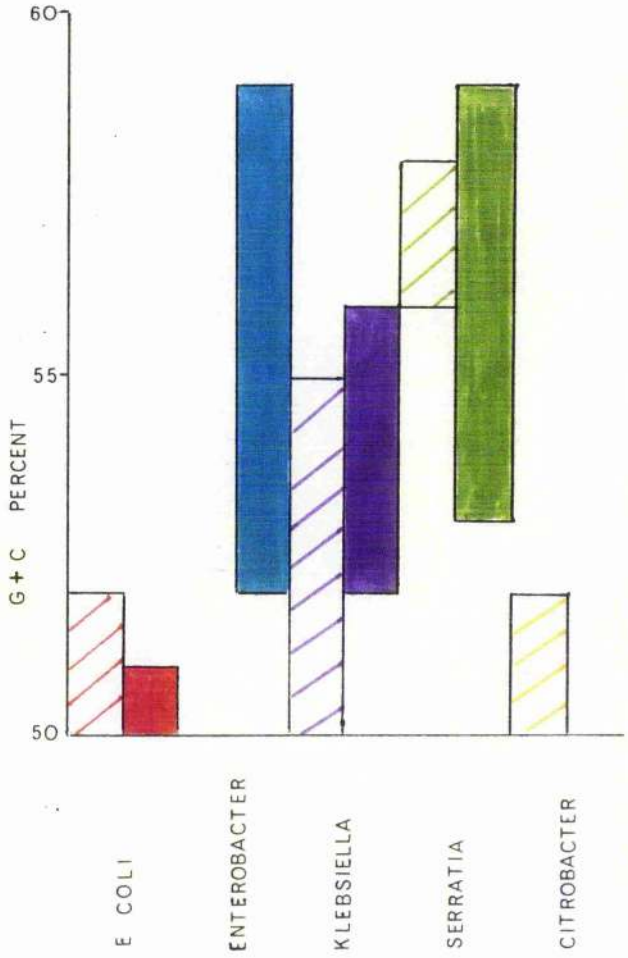
FIGURE 3.8

RELATIVE EFFICIENCY OF RESISTANCE TRANSFER FROM E COLI DONOR TO NAL-RESISTANT RECIPIENTS



G + C RATIOS FOR COLIFORMS

After: Bergey, (1974)
Wilson and Miles, (1975)



Cross hatched = Bergey, (1974)

Blocked = Wilson and Miles, (1975)

3.7. Transfer of Drug Resistance Factors from Group D Streptococci to S. faecalis JH2-2 Recipient.

The results of the transferability and frequencies of transfer from multiple-resistant strains of group D streptococci to the plasmid-free S. faecalis JH2-2 are shown in Table 3.12. The results obtained suggest plasmid involvement in the transference of drug resistance between the group D streptococci. The frequency of transfer varied between 0.07×10^{-7} - 0.53×10^{-1}. All of the isolates transferred at least two of their resistance determinants. Two strains with resistance patterns ApPnSmTc and ApPnSmEmTc respectively, transferred all markers except Ap and in both cases, the transfer frequency of Pn was very low. Sm could not be transferred from two strains with the resistance pattern SmEmTc. When the donor strains were haemolytic, both the drug resistance factors and the haemolytic activity were simultaneously transferred.

The plasmid-free JH2-2 S. faecalis recipient accepted drug resistance factors not only from other donor strains of multiple resistant S. faecalis but in addition from two S. faecium strains. The frequencies of transfer from S. faecium to S. faecalis JH2-2 in general, were not significantly lower than those between S. faecalis and S. faecalis. One may have expected a differential in transfer between the two species as was the case in the coliforms.

3.8. Plasmid Curing

3.8.1. The Effects of Curing Agents on the Coliforms

The results of the effects of storage, incubation at a high temperature and a chemical agent on the stability of drug resistance factors in the coliforms is presented in Table 3.13. During storage at room temperature in the dark, 5 out of the 7 different resistance pattern groups tested, lost part of their markers. There was a simultaneous

TABLE 3.12 TRANSFERABILITY OF DRUG RESISTANCE MARKERS FROM MULTIPLE RESISTANT GROUP D STREPTOCOCCI TO S. FAECALIS JH 2-2 RECIPIENT

Donor Strain	Resistance Markers	Markers Transferred	Transfer frequency of Drug
DS5 S. faecalis	Em Tc [1]	Em	0.27×10^{-6}
		Tc	0.43×10^{-4}
SB69 S. faecium	Ap Pn Sm Tc	Pn	0.20×10^{-6}
		Sm	0.15×10^{-6}
		Tc	0.22×10^{-5}
SB94 S. faecalis	Sm Em Tc	Sm	0.08×10^{-5}
		Em	0.13×10^{-3}
		Tc	0.10×10^{-3}
K46 S. faecium	Ap Pn Sm Em Tc	Pn	0.11×10^{-6}
		Sm	0.09×10^{-6}
		Em	0.11×10^{-4}
		Tc	0.11×10^{-4}
K55 S. faecalis	Sm Em Tc	Em	0.41×10^{-6}
		Tc	0.15×10^{-6}
K60 S. faecalis var. liquefaciens	Sm Em Tc	Sm	0.10×10^{-3}
		Em	0.53×10^{-1}
		Tc	0.31×10^{-3}
K87 S. faecalis var. zymogenes	Sm Em Tc [1]	Sm	0.47×10^{-7}
		Em	0.12×10^{-5}
		Tc	0.10×10^{-5}
K88 S. faecalis var. liquefaciens	Sm Em Tc	Em	0.07×10^{-7}
		Tc	0.14×10^{-7}

1 haemolytic strains

All matings were carried out for 4h and the transconjugants then plated on appropriate selective agar.

TABLE 3.13 EFFECT OF CURING AGENTS ON THE STABILITY OF MULTIPLE DRUG RESISTANT COLIFORMS

Strain	Treatment	Colonies No.tested expt.	/% susceptible to: Ap	Cx	Sm	Tc	Su	Strain	Treatment	Colonies No.tested/ expt.	%susceptible to: Ap	Cx	Sm	Tc	Su
CB18	Storage at 20°C for 6 months	300	0	0	100	NT	NT	CB80	Storage at 20°C for 6 months	300	100	NT	100	NT	1
	Incubation in broth at 45°C	300	0	0	0	NT	NT		Incubation in broth at 45°C	300	NT	NT	100	NT	3
	Incubation in AO broth at 25µgml^{-1}	300	0	0	0	NT	NT		Incubation in AO broth at 25µgml^{-1}	300	NT	NT	100	NT	100
	Control	300	0	0	0	NT	NT		Control	300	NT	NT	NT	NT	0
CA22	Storage at 20°C for 6 months	300	0	0	NT	NT	0	CB81	Storage at 20°C for 6 months	300	NT	NT	100	NT	.66
	Incubation in broth at 45°C	300	0	0	NT	NT	0		Incubation in broth at 45°C	300	NT	NT	NT	NT	4
	Incubation in AO broth 25µgml^{-1}	300	0	0	NT	NT	0		Incubation in AO broth 25µgml^{-1}	300	NT	NT	NT	NT	8
	Control	300	0	0	NT	NT	0		Control	300	NT	NT	NT	NT	0

AO = Acridine orange

NT = Not tested

TABLE 3.13 EFFECT OF CURING AGENTS ON THE STABILITY OF MULTIPLE DRUG RESISTANT COLIFORMS

Strain	Treatment	Colonies No. tested/% susceptible to: expt.	Ap	Cx	Sm	Tc	Su	Strain	Treatment	Colonies No. tested/% susceptible to: exp.t	Ap	Cx	Sm	Tc	Su
CB49	Storage at 20°C for 6 months	300	NT	NT	0	0	0	CB93	Storage at 20°C for 6 months	300	0	100	100	0	0
	Incubation in broth for 45°C	300	NT	NT	0	.66	0		Incubation in broth for 45°C	300	0	NT	NT	.33	0
	Incubation in AO broth 25µgml^{-1}	300	NT	NT	0	.66	0		Incubation in AO broth 25µgml^{-1}	300	0	NT	NT	.33	0
	Control	300	NT	NT	0	0	0		Control	300	0	NT	NT	0	0
CB28	Storage at 20°C for 6 months	300	0	100	0	NT	0								
	Incubation in broth at 45°C	300	0	NT	0	NT	0								
	Incubation in AO broth 25µgml^{-1}	300	0	NT	0	NT	0								
	Control	300	0	NT	0	NT	0								

AO = Acridine orange

NT = Not tested

loss of two drugs in two isolates, and in either case Sm was involved. Total loss of Cx and Ap, and partial loss of Su was observed. In all these cases the level of resistance to the drugs involved was low. Incubation at an elevated temperature cured four isolates of their resistances. Treatment with acridine orange broth at 25 μgml^{-1} did not cause any appreciable loss and this may suggest that the resistance factors were very stable.

3.8.2. The Effects of Curing Agents on the Group D Streptococci

With respect to the group D streptococci, no spontaneous loss of drugs other than Ap was observed. Two isolates exhibiting simultaneous resistance to Ap and Pn spontaneously lost their resistance to Ap only. Treatment with acridine orange broth at 10μgml^{-1} or incubation at an elevated temperature caused a partial loss of all markers. This partial loss suggests the stability of the plasmid. Table 3.14 shows the results of the curing experiment on the group D streptococci.

TABLE 3.14 EFFECT OF CURING AGENTS ON THE STABILITY OF MULTIPLE DRUG RESISTANT STREPTOCOCCI

Strain	Treatment	Colonies No. tested/expt.	% susceptible to: Ap	Pn	Sm	Em	Tc
DS5	Storage at 4°C for 6months	300	NT	NT	NT	0	0
	Incubation in broth at 45°C	300	NT	NT	NT	1.66	2.3
	Incubation in AO broth 10μgml^{-1}	300	NT	NT	NT	1.66	2.3
	Control	300	NT	NT	NT	0	0
SB69	Storage at 4°C for 6months	300	100	.	.	NT	100
	Incubation in broth at 45°C	300	100			NT	1.66
	Incubation in AO broth 10μgml^{-1}	300	0	0	0	NT	1·66
	Control	300	0	0	0	NT	0
SB94	Storage at 4°C for 6months	300	NT	NT	NT	0	0
	Incubation in broth at 45°C	300	NT	NT	NT	2.6	2.6
	Incubation in AO broth 10μgml^{-1}	300	NT	NT	NT	2.6	2.6
	Control	300	NT	NT	NT	0	0
K46	Storage at 4°C for 6months	300	100	NT			
	Incubation in broth at 45°C	300	NT	NT			1.66
	Incubation in AO broth 10μgml^{-1}	300	NT	NT			1.66
	Control	300	NT				0

AO = Acridine orange

NT = Not tested

TABLE 3.14 EFFECT OF CURING AGENTS ON THE STABILITY MULTIPLE DRUG RESISTANT STREPTOCOCCI

Strain	Treatment	Colonies No. tested / % susceptible to: expt.	Ap	Pn	Sm	Em	Tc	Strain	Treatment	Colonies No. tested / % susceptible to: expt.	Ap	Pn	Sm	Em	Tc
K55	Storage at 4°C for 6months	300	NT	NT			0	K87	Storage at 4°C for 6months	300	NT	NT	0	0	0
	Incubation in broth at 45°C	300	NT	NT			1		Incubation in broth at 45°C	300	NT	NT	0	0	0.33
	Incubation in AO broth $10\mu gml^{-1}$	300	NT	NT			2		Incubation in AO broth $10\mu gml^{-1}$	300	NT	NT	0	0	2.3
	Control	300	NT	NT	0	0	0		Control	300	NT	NT	0	0	0
K60	Storage at 4°C for 6months	300	NT	NT			0	K88	Storage at 4°C for 6months	300	NT	NT			0
	Incubation in broth at 45°C	300	NT	NT			2.3		Incubation in broth at 45°C	300	NT	NT			1
	Incubation in AO broth $10\mu gml^{-1}$	300	NT	NT			2.3		Incubation in AO broth $10\mu gml^{-1}$	300	NT	NT			1.66
	Control	300	NT	NT			0		Control	300	NT	NT			0

AO = Acridine orange

NT = Not tested

Electron micrographs of plasmid DNA isolated from selected multiple resistant Streptococci. DNA molecules were stained with uranyl acetate.

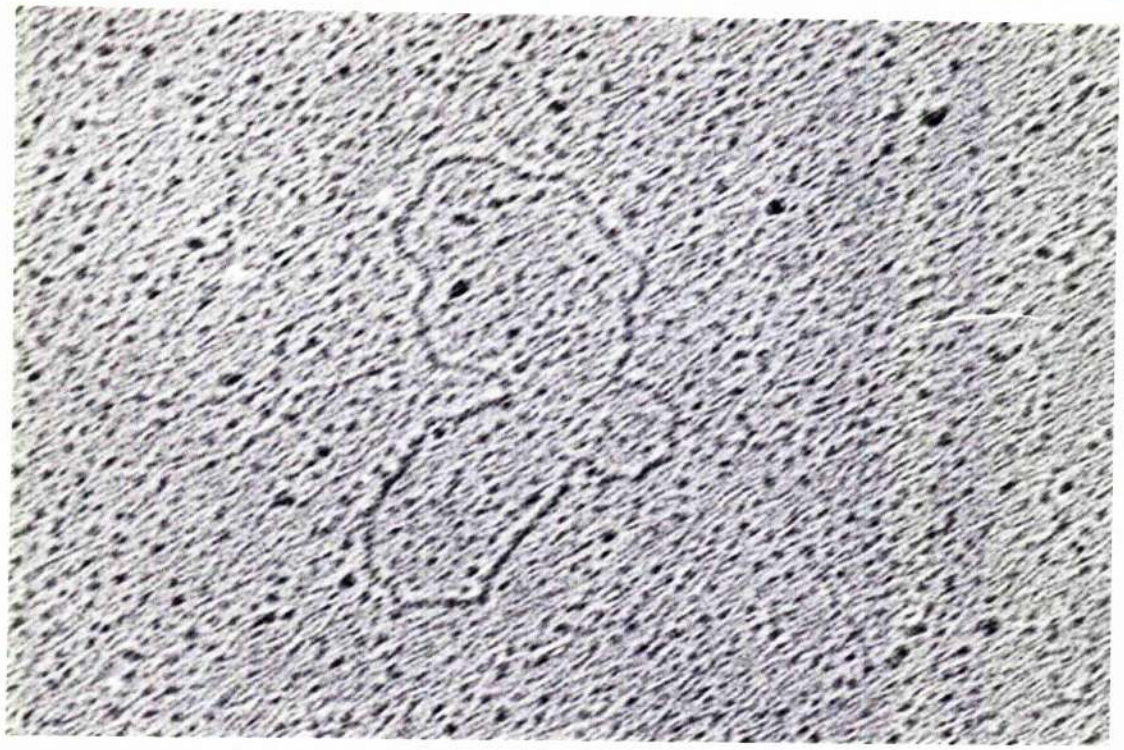


Plate 3.1 SW 69H, at 90,000 x 8 magnification.

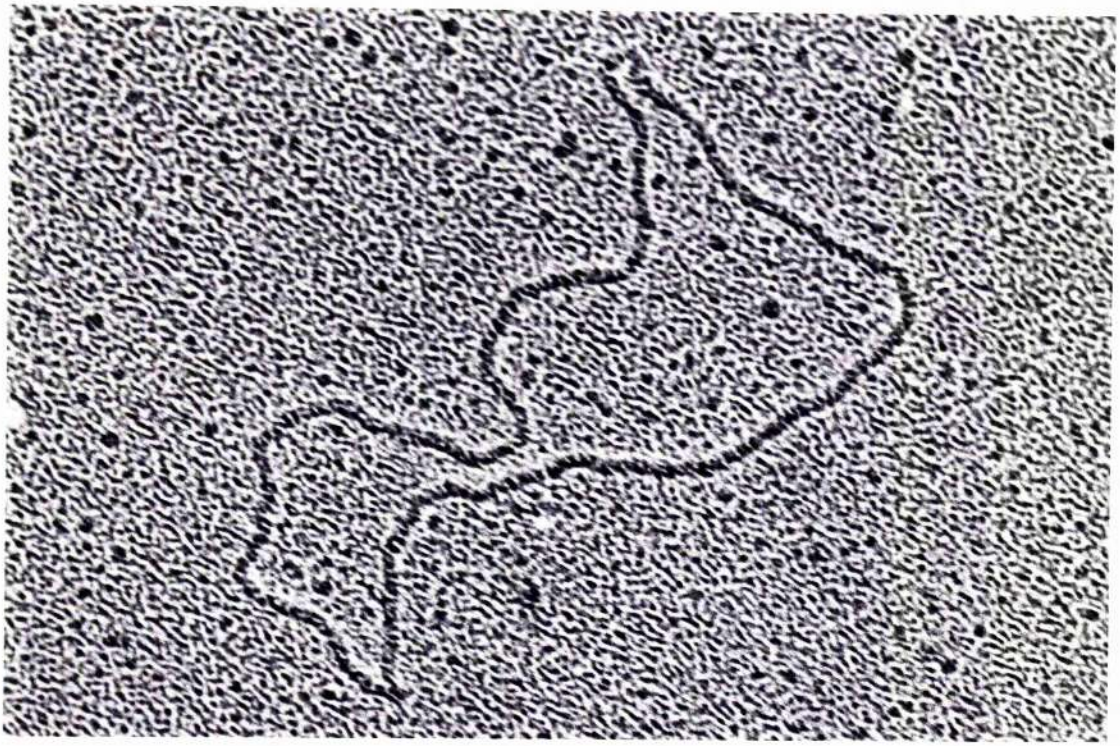


Plate 3.2 K 46L, at 90,000 x 10 magnification.

Electron micrographs of plasmid DNA isolated from selected multiple resistant Streptococci. DNA molecules were stained with uranyl acetate.

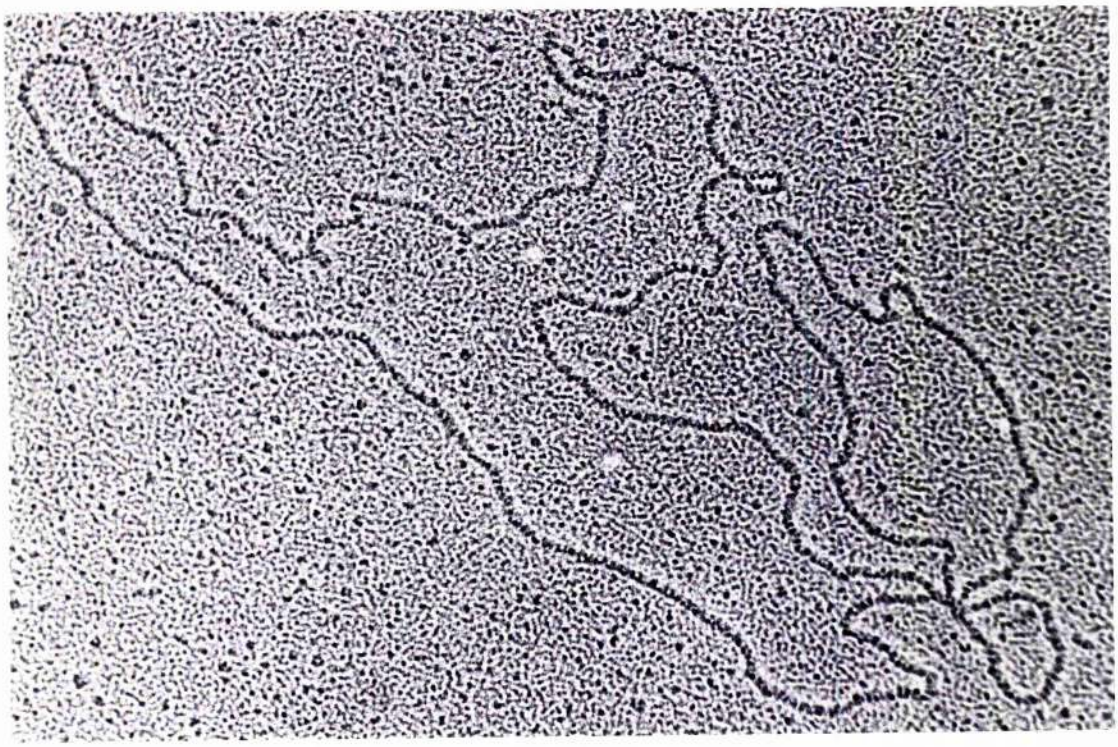


Plate 3.3 K46H, at 90,000 x 7.7 magnification.

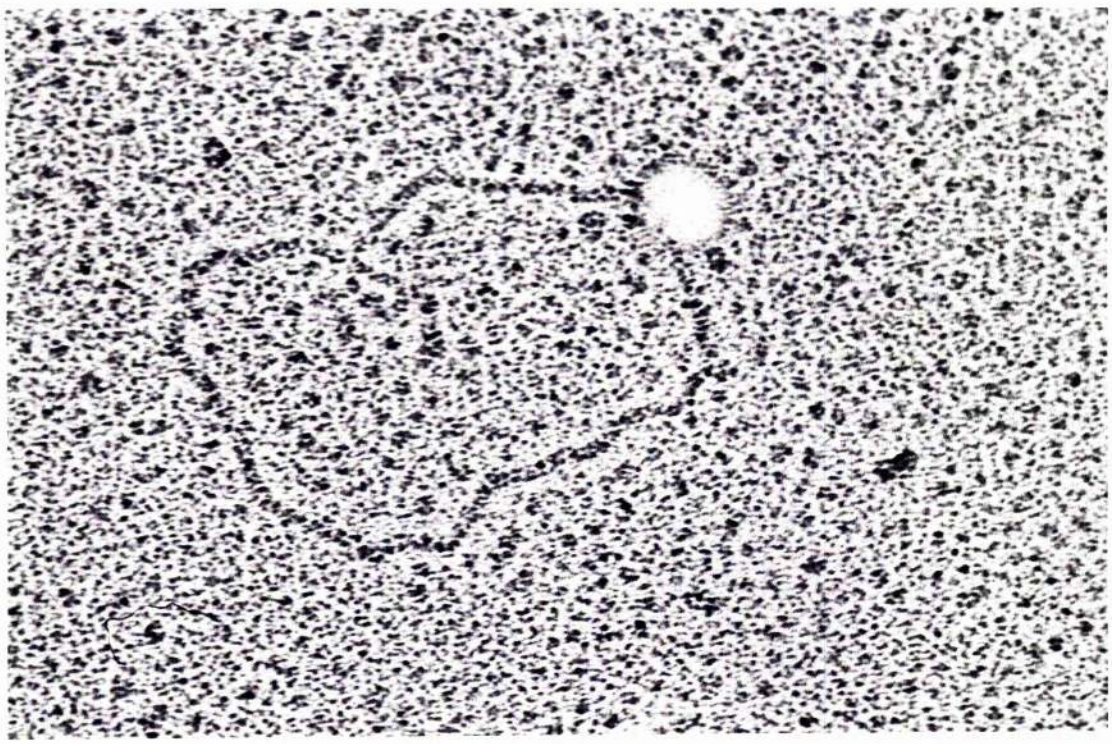


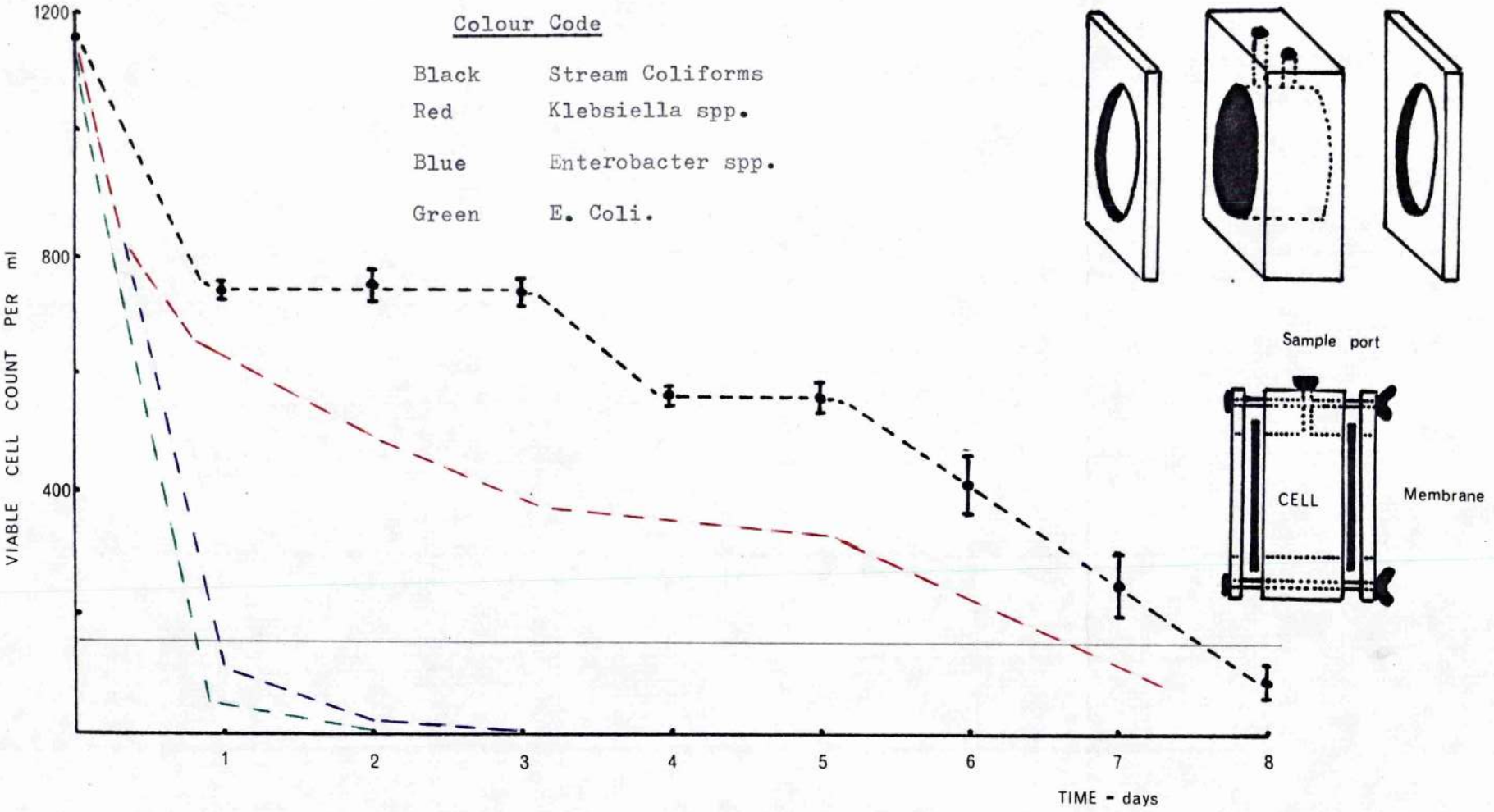
Plate 3.4 SW 94L, at 90,000 x 15.2 magnification.

3.9. Demonstration of the Presence of Plasmids

Whereas a great deal is known about plasmids (R-factors) from gram-negative bacteria, especially the enterobacteria, significantly less is known from the group D streptococci. It was only recently that conjugal transfer between the group D streptococci was demonstrated (Jacob and Hobbs, 1974; Van Embden et al., 1977; Marder and Kayser, 1977; Engel et al., 1980). For this reason, an attempt at the isolation of plasmids from multiple-resistant coliforms was not made. Instead, attention was focussed on possible plasmids from group D streptococci within the time limit available.

Caesium chloride-ethidium bromide gradient centrifugation on three of the multiple-resistant group D streptococci showed that each contained a cccDNA that banded separately from chromosomal DNA. Two bands were seen: a low band and a high band. Plasmid fractions were collected and stored at $4^{o}C$. Preliminary agarose gel electrophoresis of the fractions indicated the presence of plasmids, but due to lack of equipment photographs of the gel were not obtained. Notwithstanding this difficulty, plasmid size was determined by electron microscopy. Plasmid DNA was stained with uranyl acetate and electron micrographs determined contour length. From these measurements the molecular size of the plasmids were estimated and were found to vary between 5 and 25 megadaltons. The MW of the low band of SB69 was found to be 8×10^6 and the high 10×10^6. The low band of K46 was 10×10^6 and the high 25×10^6. SB94 had a low band which was found to be 5×10^6 (Plates 3.1 - 3.4).

FIGURE 3.9 SURVIVAL CURVES FOR COLIFORM BACTERIA



3.10. Survival of Coliform Bacteria in Stream Water

The results of the die-off rates of coliforms present in stream water and those of the pure strain cultures are shown in Figure 3.9. The appearance of the curve for the stream water sample in Figure 3.9 suggested that certain members of the coliform group may have a different survival rate from others. The results obtained with the pure cultures would appear to confirm this view. In terms of persistence, Klebsiella spp. appeared to persist much longer than either E. coli or Enterobacter spp., and that E. coli and Enterobacter spp., appeared to have the same survival rate. Time did not permit investigation of survival rates of Citrobacter or Serratia species in water or to investigate what effect known mixed cultures of these genera had on survival.

3.11. Survival of Anaerobes during Sewage Treatment

Since anaerobes by far outnumber any other group of bacteria in human faeces, an investigation of their survival during passage through the sewage treatment plant was initiated. The results of the enumeration of the obligate anaerobic bacteria and of the microaerophilic bacteria, isolated on the various selective media and their presumptive identification are presented in (Table 3.15). The results suggest that substantial numbers of obligate anaerobic bacteria could withstand the aerobic treatment process. From the primary tank, with the exception of the counts recorded on Perfringens agar, at least 99% of the bacteria were obligate anaerobes whereas in the final effluent, between 0.01 - 18.8% were microaerophiles. There was a variation in count on the various media. This difference in count may reflect the degree of selectivity of the different media. Some of the media, for

Table 3.15 Survival of anaerobes during passage through the sewage treatment plant.

Medium.	Count.ml^{-1} ($N_2/H_2/CO_2$) Primary tank (A)	Count.ml^{-1} (10% CO_2) Primary tank	Count.ml^{-1} ($N_2/H_2/CO_2$) Effluent (B)	Count.ml^{-1} (10% CO_2) Effluent	No. of colony types (A)	No. of colony types (B)	Gram stain (A)	Gram stain (B)	Type of colonial appearance/morphology (A)	Type of colonial appearance/morphology (B)	Presumptive identification.
Kanamycin	4.8-5.3 x 10^6	3.0-4.8 x 10^4	1.6 x 10^5	3.0 x 10^3	4	4	+	+	1	1	Clostridia
							+	+	2	2	?
							+		3		Peptococci/Peptostreptococci.
							+		4		
								-		5	?
								-		6	?
Neomycin/	5.2-6.6	1.2 x 10	5.8 x	1.2 x	3	3	-		7		?
Vancomycin	x 10^5	-1.0 x 10^3	10^4	10			-	-	8	9	Fusobacteria.
							-	-	10	10	Bacteroides/Fusobacteria.
								-		11	?
Neomycin (Unboiled)	2.3-7.6 x 10^6	3.7 x 10 -8.8 x 10^3	1.3 x 10^5	9.9	4	4	+	+	1	1	Clostridia.
							+	+	12	12	Peptococci/Peptostreptococci.
							+		4		
							-	-	8	8	Bacteroides/Fusobacteria.
								-		13	?
Neomycin (Boiled)	1.2 x 10^2	0	4.9 x 10	0	2	2	+	+	1	1	Clostridia (sporulated).
							+	+	14	14	
Kanamycin/ Bile	7.0-8.1 x 10^5	5.2 x 10^2 -2.5 x 10^3	7.4 x 10^3	5.4 x 10^2	1	2	-	-	8	8	Bacteroides fragilis
								-		10	

Table 3.15.(cont.).

Medium	Count.ml^{-1} ($N_2/H_2/CO_2$) Primary tank (A)	Count.ml^{-1} (10% CO_2) Primary tank	Count.ml^{-1} ($N_2/H_2/CO_2$) Effluent (B)	Count.ml^{-1} (10% CO_2) Effluent	No. of colony types (A)	No. of colony types (B)	Gram stain (A)	Gram stain (B)	Type of colonial appearance/morphology (A)	Type of colonial appearance /morphology (B)	Presumptive identification
Rifampin	3.0-4.8 x 10^5	0-1.3 x 10^2	1.3 x 10^3	1.3 x 10^2	2	1	-		8		Fusobacteria
							-	-	10	10	
Perfringens agar (Unboiled)	4.5 x 10^3-3.0 x 10^4	8 x 10^3			4		+		15		Clostridia
							+		12		Peptococci/Peptostreptococci
							+		4		Peptococci/Peptostreptococci/Strep. faecalis*
							-		9		Bacteroides/Fusobacteria
Perfringens agar (Boiled)	1.1 x 10^2	3			2		+		15		Clostridia (sporulated).
							+		14		

Colonial appearance/morphology : (1): Large mustard-brown colonies; square-ended bacilli; (2): Small creamy-white colonies; long thin bacilli; (3): Dark centred colonies with light surround; cocci, often in pairs; (4): Medium creamy-white colonies; cocci; (5): Large flat greenish colonies; pleomorphic bacilli; (6): Small yellow domed colonies; coccobacilli; (7): Brownish colonies with opaque surround; long bacilli; (8) Dark centred colonies with clear surround; normal and pleomorphic bacilli; (9): Yellow domed colonies, variation in size; pleomorphic bacilli; (10): Creamy colonies; pleomorphic bacilli; (11): Pearl-drop colonies; pleomorphic bacilli; (12): Pearl-drop colonies; cocci, sometimes in pairs; (13): Very small colonies; bacilli; (14): Creamy colonies; square-ended bacilli; (15): Black centred colonies with opaque surround; square-ended bacilli.

*Occasional strains of Streptococcus faecalis may grow (Oxoid Ltd., 1980).

example Nm-blood agar, selects for a wide variety of anaerobes e.g. bacteroides, fusobacteria, veillonella, clostridia, gram-positive anaerobic cocci and gram-positive non-sporing anaerobic bacilli. Other media, such as Km-bile or Rif-blood agar, are highly selective for Bacteroides fragilis and fusobacteria respectively. High counts were obtained for bacteroides and fusobacteria. The boiled sample of sporulating clostridia revealed approximately equal numbers of clostridial isolates on both Nm-blood agar and Perfringens agar.

4. DISCUSSION

4. DISCUSSION

4.1. Viable Counts

The results of the viable counts (Table 3.1) show a day-to-day variation throughout the sampling periods. This may be due to uncontrollable environmental factors.

The viable bacterial numbers obtained from the water samples can best be summarized as follows: primary settling tank > humus settling tank > below sewer outfall > above sewer outfall. The increase in coliforms and group D streptococci in samples from below the outfall ostensibly measures the degree of contamination by the input of sewage effluent into the water course.

The approximate 36-fold increase in coliforms in samples from below the sewer outfall compared with above the outfall, is less than that reported by Hughes and M^{c}ynell (1974), whose counts were recorded above and below a sewer outfall on the River Stour in Kent. This difference may be due to the population the treatment plant was designed to serve, and to the relationship between the volume of effluent discharged and the water flow in the water course into which the effluent is discharged.

The approximate 150-fold increase in group D streptococci in samples from below the outfall may suggest that the major contribution of group D streptococci to the water course is from human wastes.

The major contributory factors to the coliform levels at the village sampling point were (a) agricultural drain-off since it is known that coliforms survive in soil (Medrek and Litsky, 1960), (b) the input from septic tanks and (c) the known use of sludge digestion solids as fertiliser on the surrounding agricultural land, and this is probably the major factor. Water Authorities dispose of treated or untreated

sewage sludge on agricultural land as a source of nutrients and humus (Technical Report TR71, 1978). Potentially pathogenic bacteria have been isolated from sludges (Jones et al., 1980). Such disposal of sewage sludge will certainly increase the number of pathogenic and non-pathogenic microorganisms in the environment and may present a danger to public health. On the other hand, sewage sludge represents a readily available source of organic matter for use as fertiliser, but there would thus be a great loss of potential fertiliser if its use was limited. Thus widespread use of sewage sludge must be properly managed so that the quality of life is not affected.

It is assumed that the intention of Water Authorities is presumably not a Pyrrhic one (to vanquish the human population) but is to live in harmony with it. To attain this goal, the external environment must be skillfully managed. Uncontrolled disposal of sewage sludge on the environment may not only cause grave danger to public health, but may also deny future generations the chance to choose the kind of environment that they would wish to live in.

The recommended media for the isolation of viable coliforms or group D streptococci in water samples (D.H.S.S. (Welsh Office), 1969) contain an appropriate selective agent. Even though viable counts appear to assess the number of "living" bacteria, no comprehensive definition of "living" is available. It is generally accepted that the ability to form on a solid medium colonies visible to the naked eye, or to produce visible turbidity in a broth culture, is a verification of viability. On a solid medium, 'stressed' or 'injured' organisms may be unable to form visible colonies. This debilitation of cells has been observed in coliforms (Bissonnette et al., 1975) suspended in fresh water. It follows that 'injured' cells would automatically be excluded in a plate count. In addition, when intestinal organisms have been

exposed to the extra-enteral conditions, their isolation on selective media discriminates against those that have, stress factors apart, lost the ability to perform a function such as the fermentation of lactose or the reduction of tetrazolium chloride. Consequently, the true density of such bacteria in water cannot be accurately measured. For these reasons, colony counts appear to have no absolute significance but serve as an indication of the number of organisms able to form colonies at that time on some artificial medium.

4.2. Model Media

Media for increased recovery of organisms have been proposed. To achieve such a medium Dufour and Cabelli (1974) tested a variety of fermentable carbohydrates, and lactose was found to be the most effective for the differentiation of coliforms. It is not surprising that despite the suggestions of Mackie (1913) and of Mossel (1957) for the replacement of lactose with mannitol, lactose still remains the sugar of choice in the primary isolation medium for coliforms. The major advantage of lactose over mannitol is that pathogens can be generally differentiated from non-pathogens by their lactose reaction (although lactose-negative non-pathogens are known).

The ideal medium should have the attribute of permitting the recovery of the same number of both stressed and non-stressed organisms that would grow on a non-selective or enriched medium. This ideal medium should also be specific enough to recover only the desired organisms, selective enough for their total recovery and finally, it should inhibit the background overgrowth of other unwanted organisms. So far no ideal selective medium is available that would fit the above criteria.

Attempts at achieving this ideal status have been made recently, notably by Dufour and Cabelli (1974) for coliform isolation and by

Abshire (1977) and by Donnelly and Hartman (1978) for group D streptococci isolation. The odd choice of some of the constituents of these media is one drawback, the other is the difficulty of acceptance of such media into widespread use so as to replace the well-known and time-tested media currently used in water bacteriology.

It is reasonable to be aware of the presence of 'stressed' or "injured" bacteria in water. These organisms do not deserve too much emphasis in practical water bacteriology. They are usually outnumbered by viable organisms and contribute very little to indicate hazardous contamination of water. Moreover, they are not the last bastion of water quality. What constitutes real danger to public health is the presence in water of living bacteria not dead or 'stressed' cells. However, stressed cells, although they may not grow on selective media, may be quite viable as pathogens in the animal body. As a recognition of the presence of 'stressed' cells a resuscitation period of incubation is usually employed. This period conditions these 'stressed' cells for growth on the selective media.

4.3. Selectivity of Media

Slanetz and Bartley's medium has been reported as being very selective for group D streptococci, and when incubated at an elevated temperature (44° - 45°C) all red or maroon colonies may be accepted as faecal streptococci (Taylor and Burman, 1964; Mead, 1966; D.H.S.S. (Welsh Office), 1969). Comparison of KF streptococcus agar with this medium on water and sewage samples show the superiority of Slanetz and Bartley's medium over KF. Raibaud et al., (1961), on the contrary, observed that Slanetz and Bartley's medium was not totally selective for group D streptococci as lactobacilli overgrew the group D streptococci in their study of pig caecal samples. Pavlova et al., (1972), in their

study of group D streptococci in faeces, sewage and food samples, also reported that 18.4% of the organisms growing on this medium were not group D streptococci. More recently, Burman et al., (1978) and Noble, (1978) claimed that this medium was not 100% selective for the group D streptococci. It is interesting to note that Noble, (1978) incubated his plates at 37°C. Had Noble incubated his plates at the recommended temperature, one would have supposed that more group D streptococci would have been isolated. The disadvantage of this medium it has been claimed, is the inhibitory effect of the azide on S. bovis (Sabbaj et al., 1971; Switzer and Evans, 1974). The results from this study indicate that the selectivity of the medium was indeed specific. Although the percentage of S. bovis recovered was low, this may be due to their rapid die-off rate. McFetters et al., (1974), have shown that S.bovis and S. equinus die-off faster than S. faecalis and S. faecium. Furthermore, none of the group D streptococci strains failed to tolerate 40% bile + 0.1% aesculin.

The reliability of METB, on the other hand, has not been subjected to wide criticism provided the recommended standard grade of Teepol 610 (B.D.H.) is used. An improvement could be obtained by gelling the medium with purified agar. The advantage of this is that colonies developing on the agar are larger and more robust than those on absorbant pad (personal observation). In addition, lactose-fermentation characteristics appear to be unequivocal on the agar medium.

4.4. Speciation of Coliform Isolates

The primary task for the early water bacteriologists had been in the identification of E. coli sensu strictu, by using the Eijkman test and indole production at 44°C. The standard IMViC tests (Parr, 1938) had been employed as an extension of this for the identification of other coliforms. Other workers examining coliforms from environmental sources have not speciated their isolates beyond the determination of the

percentage of E. coli strains present. The identification procedures for the composite members of the coliform group, employed in this study have been shown to be practicable. The premise has been that any isolate that warranted picking off was important enough to be identified. Judicious use of the tests employed in this study (Table 3.2) has allowed their identification.

All the E. coli strains identified produced acid and gas at 44°C and produced indole at that temperature. No false positives were encountered.

The IMViC tests presented some problems with both Enterobacter and Klebsiella species which nearly always gave similar patterns of reactions. The biochemical reactions that are of particular value for the identification of the different genera, except E. coli, are the invariable fermentation of glucose and the production of β-galactosidase. In general, there was a fairly close agreement with the behaviour of these species and those shown by Cowan, (1974) and by Lennette et al. (1974). Some differences in reactions require comment. According to Cowan, (1974), gluconate oxidation is a character possessed by all Enterobacter species. In this study 90% of the Enterobacter isolates above and 100% below oxidised gluconate. In addition 93% of the isolates above and 76% below utilized malonate which (Cowan, 1974) according to a test, is non-specific. Such results are suggestive that not all strains possess the ability to utilize all substrates and results should be represented as percentages instead of + or - or d as presented by Cowan, (1974). The API system recognises this and presents results as percentages.

The time is ripe for the use of other colfiroms as indices of pollution. Investigation for the presence of only E. coli is of limited use. It seems logical to attribute the same public health significance to coliform other than E. coli, as to E. coli itself.

Speciation of the coliforms (Figure 3.1) showed a fairly similar distribution of genera from both sampling points with E. coli being the predominant species. As this finding was contrary to expectation, an investigation of the percentage of E. coli strains in (a) the effluent from the second primary tank and (b) the effluent from the humus settling tank was made. The results revealed that only 56% and 53% respectively of the coliforms at those points were E. coli. Such findings emphasise the importance of speciation as carried out in this study. Since E. coli is the predominant aerobe in the human intestine, one would have expected a significantly higher percentage than the 56% in the second primary tank. That this was not the case, may be due to their die-off rates in the extra-enteral environment. But if this speculation were true, one would have expected a similar reduction in the population of faecal E. coli in the effluent of the humus settling tank and at the sampling point below the outfall as observed by Fontaine and Hoadley, (1976). One intriguing possible explanation for not finding a significant reduction in E. coli may be that after the initial reduction of their number, the surviving strains spontaneously became better adapted to the prevailing conditions in the environment. Another possible explanation would be that, of these strains of bacteria at least, their resistance to antibiotics selects for their survival. A similar observation was made by Fontaine and Hoadley (1976). They showed that the proportion of antibiotic-resistant E. coli did not change appreciably during sewage treatment. In addition, it could be possible that in the location the number of bacteriophages which are specific for E. coli is very low.

Whereas above the outfall the number of Enterobacter isolates were greater than Klebsiella, below the outfall, their numbers were approximately equal. The ecology of these species, unlike E. coli,

is not only in humans and animals (see Chapter 1.2) and this may account for their occurrence at both sampling sites. The numbers of both Serratia and Citrobacter isolates were too small to draw any definite conclusions but their occurrence at both sampling sites may reflect their wide distribution in nature and their probable faecal origin.

4.5. Speciation of Group D Streptococci isolates

The identification of all the group D streptococci from water and clinical sources has been a difficult task. Many studies have been initiated but not many have shown definitive results. The scheme (Table 3.4) used for their identification in this investigation has been shown to be reliable and feasible for research purposes, and could be simplified for diagnostic laboratories.

Facklam and Moody (1970) showed that the hydrolysis of aesculin in a medium containing 40% (w/v) bile is a test of choice in the presumptive identification of group D streptococci. Facklam et al. (1974) again showed the bile-aesculin test as a reliable presumptive identification test for group D streptococci with a 99% positive result on 920 strains compared with serological tests. Gross et al., (1975) on a similar sample, found 99.7% of these organisms to be bile-aesculin-positive. Ator and Starzyk, (1976) also found 98% of their isolates as bile-aesculin-positive. Similar results were obtained in this study and the findings confirm that the hydrolysis of aesculin in the presence of bile is a specific and an excellent indicator system for bile-tolerant streptococci, when incubated for a maximum period of 48h as suggested by Facklam and Moody, (1970).

Growth at 10°C, 45°C and 6.5% (w/v) NaCl were reliable tests for distinguishing the enterococci from the non-enterococci, with the latter unable to initiate growth at 10°C or in 6.5% (w/v) NaCl. Mundt and

Graham, (1968) observed that 31.3% of their S. faecium var. casseliflavus strains failed to grow in broth at 45°C. The results of this study showed that all the strains of S. faecium var. casseliflavus grew at 45°C both in broth and on agar surfaces. The method used in the present study for their isolation from water samples required incubation at 45°C on a selective medium. Growth at 45°C was found to be a useful test in separating not only the enterococci from the non-enterococci, but also as a criterion for identifying S. faecium var. casseliflavus strains. A negative result must therefore preclude a strain from being a group D streptococcus.

Skadhauge (1950) observed that strains of S. faecalis and its variants grown in the presence of 0.04% (w/v) potassium tellurite agar produced black colonies, and other faecal streptococci did not grow at all or produced dusty-grey colonies. However, Deibel et al., (1963) noted that potassium tellurite tolerance was not specific for S. faecalis and its variants with approximately 3% of their S. faecium strains resistant to this concentration of tellurite. Facklam (1972) also found that tellurite tolerance was not 100% specific for S. faecalis and variants. Mundt and Graham, (1968) observed that the majority of their S. faecium var. casseliflavus isolates produced grey colonies on tellurite agar. Observations from this investigation showed that tellurite-tolerance was not specific for S. faecalis and variants. The percentage of S. faecalis var. faecalis from above the outfall that were tellurite-tolerant was 94%; also 4% of S. faecium strains below the outfall were tellurite-tolerant. The percentage of S. faecium strains that were tellurite-tolerant appears to be in agreement with Deibel and his collaborators (1963). With respect to the isolates of S. faecium var. casseliflavus, the results show that they were also tellurite-tolerant growing on the medium as grey colonies. The proportion of the isolates

100% that were tellurite-tolerant was higher than that of Mundt and Graham, (1968) 96%.

Barnes, (1956) showed that only S. faecalis and variants reduced 2,3,4-triphenyl tetrazolium chloride (T.T.C.) to a red formazan. Whittenbury, (1965) observed a similar finding with 100% of his S. faecalis isolates reducing T.T.C. whereas all his S. faecium strains failed to do so. Facklam, (1972), on the contrary, found that 1% of his S. faecalis strains failed to reduce T.T.C. Gross et al., (1975) rejected the use on their isolates of tellurite-tolerance and tetrazolium reduction tests on the grounds of their variability or difficulty of interpretation. Waitkins, (1978) found that the activity of T.T.C. varied from batch to batch. This led her to the conclusion that T.T.C. medium was unsatisfactory for distinguishing S. faecalis from other group D streptococci from human sources. As a result, she excluded T.T.C. from her list of tests in later studies (Waitkins et al., 1980). The results from this study showed that T.T.C. reduction was not specific for S. faecalis and variants. The proportion of T.T.C.-reducing S. faecalis var. faecalis above the outfall was 75%. In addition, 7% S. faecium and 17% S. faecium var. casseliflavus were T.T.C.-positive. Below the sewer outfall, however, 95% of the S. faecalis var. faecalis strains reduced T.T.C. In addition, 4% S. faecium and 14% S. faecium var. casseliflavus reduced T.T.C. As far as the clinical isolates were concerned, 94% S. faecalis var. faecalis were T.T.C.-positive. It is however, germane to note that T.T.C. at a concentration of 0.1% (w/v) was not reduced by any S. bovis, S. equinus or S. durans strain.

Microorganisms that ferment sorbitol, produce L-tyrosine decarboxylase and reduce T.T.C. in the presence of thallous acetate (Mead, 1963) have been accepted as S. faecalis sensu strictu. Burman et al., (1978) accepted this phenomenon as a definitive identification of S. faecalis and variants. The results from this investigation suggest

that this test is not specific for S. faecalis. Not all the S. faecalis isolates were positive on this medium. In addition, 7% S. faecium and 17% S. faecium var. casseliflavus from above the outfall grew on this medium. Below the outfall, 97% of S. faecalis var. faecalis were positive whereas 95% of S. faecalis isolates from clinical sources gave a positive result. The difficulty in the preparation of this medium coupled with the variability of the results does not warrant its use as a reliable test.

Pownall, (1935) studied a motile streptococcus that produced a greenish pigment on blood agar. Graudal, (1951) also using blood agar, studied yellow pigmented group D streptococci. Mundt and Graham, (1968) again studied a number of yellow pigmented streptococci from plants and coined the name S. faecium var. casseliflavus. Mundt and Graham, (1968) observed yellow pigment production on 5% (w/v) sucrose agar. The results from this study show that on blood agar S. faecium var. casseliflavus strains were characteristically yellow pigmented. Pigmentation did not necessarily require the addition of sucrose to the agar medium. Some of the pigmented strains showed a greenish type of haemolysis, and one strain was motile. These organisms may be similar to that studied by Pownall, (1935). Pigmentation was enhanced by allowing the blood plates to stand in contact with air on the laboratory bench for approximately 2h after aerobic incubation. None of the pigmented strains studied in this investigation were variants other than S. faecium var. casseliflavus. It is interesting to note that, among the strains obtained from human sources, one was pigmented. This finding may indicate that pigmented strains of group D streptococci may also originate from human sources.

The addition of manganous sulphate to an agar medium enhanced the

pigmentation of S. faecalis and its variants (Jones et al., 1963). This test was not 100% specific for S. faecalis. Above the outfall 81% S. faecalis, 50% S. faecalis var. liquefaciens and 3% S. faecium var. casseliflavus produced apparent pigment. Below the outfall, 87% of S. faecalis var. faecalis and 28% S. faecalis var. casseliflavus also produced apparent pigment. That S. faecium var. casseliflavus strains produced apparent pigment is not surprising since it produced pigment on blood agar. One would have expected all the S. faecium var. casseliflavus to have done so.

Group D streptococci have been identified using various serological methods. Recently the co-agglutination method has come into prominence with various commercial preparations in the market. Little importance has been attached to serology in recent years. To quote Cowan (1974) "Serology is no longer the final arbiter in streptococcal taxonomy, just one character to be considered with others from morphology, physiology and biochemistry". The production of potent group D antisera had been a problem. During this investigation, all the strains of group D streptococci reacted with coated staphylococci by the co-agglutination method. There were varying degrees of reaction ranging from weak to very strong. This reflects the problems encountered in the commercial preparations of antisera for group D streptococci.

On examination of the plethora of fermentation tests, together with the hydrolysis of arginine, that were employed in this study, some tests may be singled out for their dependability. These are the fermentation of mannitol, pyruvate, arabinose, melezitose, melibiose, lactose and the hydrolysis of arginine. Others may be rejected for their variability and unreliability; those are glycerol, sorbitol and inulin.

With regard to the reactions of S. faecalis var. faecalis strains to the dependable tests (except pyruvate fermentation and arginine hydrolysis) it is difficult to make any meaningful comparison with the results of other workers such as Facklam, (1972); Gross et al., (1975) and Parker and Ball, (1976). This is because S. faecalis var. faecalis is invariably grouped under the heading of either S. faecalis and variants or S. faecalis which may include the variants. As far as the fermentation of pyruvate and the hydrolysis of arginine are concerned Gross et al., (1975) showed that these tests are dependable and useful for the differentiation of S. faecalis from other group D streptococci. Waitkins, (1978) compared pyruvate fermentation with arginine hydrolysis in her study on the differentiation of S. faecalis from other group D streptococci and observed similar results to those of Gross et al., (1975). Using a shortened identification scheme for the group D streptococci, Waitkins and her collaborators (1980) included pyruvate fermentation and arginine hydrolysis as tests of choice. Their results showed that pyruvate fermentation was specific for S. faecalis. The results of the present study corroborate those of the above workers. S. faecalis and its variants readily fermented pyruvate and hydrolysed arginine whereas none of the other species possessed the property of pyruvate fermentation.

The percentages of S. faecium identified in this study which fermented arabinose (another of the dependable tests) were 100% above and below the outfall. When these figures were compared with the 96% of Facklam, (1972), 100% of Gross et al., (1975) and 100% of Waitkins et al., (1980), they indicate that virtually all S. faecium ferment arabinose. The proportion of S. faecium identified as fermenting melibiose, 86% above and 81% below is also comparable to the 87% of Gross et al., (1975). Similarly, the 100% above and 100% below, of arginine-hydrolysing

S. faecium, compared with the 100% of Gross et al., (1975) and 100% of Waitkins et al., (1980). These results indicate the unequivocal capability of S. faecium species to give positive results to these tests and are therefore dependable for their identification.

The proportion of S. faecium var. casselflavus identified in this study that gave a positive reaction to inulin was 17% above and 28% below the outfall. These figures, compared with those of Mundt and Graham, (1968), showed considerable variability. Inulin, is therefore an unreliable carbohydrate for the identification of S. faecium var. casselflavus. Furthermore, this variant has been shown to be biochemically related to both S. faecalis and S. faecium. This relationship has been illustrated in the results section (3.2.3).

The S. durans strains identified in this study had characteristics similar to those described by other workers (Facklam, 1972; Gross et al. 1975; Waitkins et al., 1980). The percentage of strains (100% above and below the outfall), fermenting lactose were similar to those of other workers quoted above. In addition arginine hydrolysis results were similar to those of Gross et al., (1975) and Waitkins et al., (1980).

These two tests, it may be noted, belong to the group of dependable tests mentioned earlier. Although melibiose fermentation is among the dependable tests, the results show that it is not a test of choice for their identification but could be used as an adjunct to other tests. In addition, the haemolytic activity of S. durans and its metabolic inactivity stand out in their identification.

The relatively small number of S. bovis strains identified in this study does not warrant much comment on their metabolic activities. The ability of this species to hydrolyse starch and ferment melibiose and lactose may be an important characteristic. But its inability to ferment pyruvate and hydrolyse arginine, although negative characteristics, may

prove sufficient when used with other tests to identify this species. The results in this study show that 50% of the isolates were unable to ferment lactose. One may assume that they may belong to one or other of the S. bovis I or II subspecies reported (Facklam, 1972; Parker and Ball, 1976). As far as S. equinus strains were concerned, only one was identified. Therefore, its fermentation reactions do not deserve any comment. But it is important to mention that its metabolic activities differed from all the other species identified and it fitted the description given for S. equinus.

With respect to anaerobic fermentation of glycerol, Gunsalus, (1947) observed that this phenomenon was characteristic of S. faecalis and its variants. The results of this study showed that although 100% of S. faecalis and its variants below the outfall, 94% of S. faecalis, 100% of the variants liquefaciens and zymogenes respectively above the outfall, possessed this characteristic, a significant percentage of S. faecium and variant casseliflavus strains were also positive. As far as S. faecium strains were concerned, Mundt and Graham, (1968) reported 50% of their isolates to be positive. The positive reaction with species other than S. faecalis and variants may be due to oxygen diffusing into the test medium through the oil layer, and also to the problem of maintaining a low Eh throughout the incubation period. This test, therefore, gave equivocal results and should not be relied upon.

The results from the speciation of group D (Fig. 3.2) streptococci isolates from below the outfall show that S. faecalis var. faecalis is the dominant group D streptococcus. This organism has previously been reported to be the predominant group D streptococcus in human faeces (Moussa, 1965; Noble, 1978). In addition, the results from the speciation of clinical isolates in this investigation also showed that S. faecalis

var. faecalis predominates. Consequently, one can only assume that because of their predominance in the human intestine, these organisms may have found their way into the water course via the effluent of the sewage treatment plant. This assumption is in support of the findings of Bartley and Slanetz, (1960) in similar studies. S. faecalis var. liquefaciens on the other hand, has been found in association with animals, vegetation, insects and soil (Standard Methods, 1975; Ator and Starzyk, 1976). If this were the case, one would have expected to have found a significantly greater proportion of S. faecalis var. liquefaciens above the sewer outfall than below. That approximately equal numbers of this strain were identified from both sampling points may indicate their wide ecological distribution both in human and in the environment. Even the results of the speciation of strains from human sources showed that they were present in significant numbers, suggesting that they have their habitation chiefly in humans. Had this species not been grouped with S. faecalis (Facklam, 1972; Ator and Starzyk, 1976) a meaningful comparative deduction of its distribution could have been made. Facklam, (1972) expressed doubts as to whether the variants of S. faecalis need to be separated for clinical diagnostic purposes. It is rather surprising that none of the S. faecalis var. liquefaciens isolates of Ator and Starzyk, (1976) actually liquefied gelatin. For a better understanding of species distribution, this variant needs to be separated from S. faecalis proper.

With respect to the distribution of S. faecalis var. zymogenes, very little is known. Apart from the occurrence of this strain from human sources little information is available about its occurrence in the environment. Moreover, confusion has arisen as to the value of haemolytic activity in differentiating this strain since haemolysis is dependent on the type of blood used (Updyke, 1957). Deibel, (1964) suggested that the variant status of zymogenes be dropped. Jacob et al., (1975) showed that

haemolytic activity was plasmid-borne with the implication that it is an unstable character and they agreed with the suggestion of Deibel, (1964). The conjugation studies during the course of this investigation have confirmed that the haemolytic activity is plasmid-borne. The differences between S. faecalis var. faecalis and variants are the proteolytic property of the liquefaciens variant and the haemolytic activity of the zymogenes variant. These differences have made the variants vulnerable to criticism. It is apparent that the question of whether the current nomenclature of S. faecalis and its variants is to remain, is now nearer no resolution than when it was first raised. At first sight it seems strange that it should be questioned at all. There is no doubt that these variable characters are at best unstable, and the same may be true for some of the characters used to differentiate other species. Indeed, when considering conjugal transfer properties, these characters should not be overlooked as they represent non-selective markers which may serve to distinguish a donor from a recipient according to the role they play during such studies. These non-selective characters transcend any objection that the status be dropped and it is recommended that the status of these should be retained.

The distribution of S. faecium strains from above and below the outfall was quite similar. This result may indicate its distribution both in nature and in human beings. Weil-Korstanje and Winkler, (1975) found almost equal proportion of S. faecium and S. faecalis in the faeces of normal adults. Studies by Buttiaux, (1958) and Kjellander, (1960) show that S. faecium strains were more common than S. faecalis strains in human faeces. Results from other indpendent studies on clinical isolates (Moussa, 1965; Noble, 1978) indicate the contrary and show S. faecium strains to be inferior in number to S. faecalis strains. Ator and Starzyk, (1976), in studies on water samples, show more S. faecium (20.97%) than S. faecalis and variants (6.52%). The results from this study showed that S. faecium strains did not predominate in samples from either sampling sites. It is difficult to relate their occurrence to the source

of water sample and represents a "gray" area.

Speciation of the streptococci isolated from above the outfall showed that S. faecium var. casseliflavus was the dominant variant. Mundt and Graham, (1968) implied that these pigmented organisms were members of the enterococci but that they were found only on plants and vegetables and were rarely associated with human beings or animals. These statements would appear contradictory if one considers the accepted definition of an enterococcus. Since these organisms had previously been reported associated with plants (Mundt and Graham, 1968), it is now open to question as to whether they are associated with human beings. Since they constitute the majority of the isolates above the sewer outfall, this finding may support the view of Mundt and Graham, (1968) that this variant is a plant epiphyte. That they occur in much smaller numbers below the outfall may be due to (a) their dilution as the water flows down the stream, (b) the input by the sewage effluent of other microorganisms which will contribute towards an effective reduction in their numbers. Although Graudal, (1951) did not specify clearly the sources of all of his pigmented streptococci a few of them did originate from human sources. Perhaps not enough attention has been paid to these unusual variants and hence there is no recent report on their occurrence.

With regard to the distribution of S. durans strains above and below the outfall, there appeared to be no significant difference from both sampling points. Their occurrence as observed in this study appears to agree with the findings of Ator and Starzyk, (1976) from similar sites.

S. bovis strains were equally distributed above and below the outfall but none was identified among the clinical strains.

4.6. Standardization of Tests

The results of the identification tests carried out in this study, when compared with those of other workers, have shown that few tests gave reproducible results. This may be due to (a) the constituents of media which may vary from one laboratory to another and (b) the variability of the test conditions. A system whereby universally reproducible results could be achieved would be an ideal situation. Gas-liquid chromatography offers data that can be universally duplicated and therefore acceptable for the identification of anaerobic bacteria, and possibly the aerobic bacteria. Furthermore, dehydrated identification kits produced by commercial firms notably A.P.I., for the identification of the Enterobacteriaceae and now for Streptococci and anaerobic bacteria, offer the ideal situation whereby identification procedures could be reproduced universally. The advantage of the A.P.I. system is that the substrates are produced by one firm, rigourously quality-controlled, and marketed with detailed instructions and computer-indexed for ease of identification. Since it is impossible to identify all bacterial strains in one laboratory, such a system, if marketed by one company only, (there are others in the market), could come close to the almost impossible dream of a standardized system with standardized methods of identification. If such a goal were achieved, the concept of an ideal identification system that is universally accepted might come into being.

This objective may be elusive because the expense of the systems often precludes their use especially in the developing countries that can least afford them. The time may not be ripe yet, but the need for having a standardized system is great. Yet it appears that this goal cannot be achieved as the gap between the rich and poor widens.

4.7. FC/FS Ratio

The FC:FS ratios above the outfall did not vary significantly from those obtained below. Even though the FC and FS counts obtained above the outfall were low as compared with those below, the FC:FS ratios remained high. The accuracy of the continued application of this ratio as an indicator of faecal pollution is highly doubtful. It indicated pollution from human sources. Below the outfall, however, the FC and FS counts were higher. These high counts did not significantly alter the FC:FS ratios from those obtained above, except on one occasion when the ratio was 0.49. Theoretically, a ratio of 0.7 would indicate pollution from domesticated animals. One would have expected to have obtained this low ratio from above the outfall where the stream mainly runs through pastoral land, rather than from below. It is difficult to draw any definite conclusions from these ratios. One could only speculate that both human beings and animals may have contributed towards the contamination of the stream. In addition to the factors mentioned earlier (4.1), pollution may be caused by (a) ineffective sewage treatment by the sewage treatment plant and (b) grazing animals in the surrounding land. Ator and Starzyk, (1976) also came to a similar conclusion in their study on bacteriological contamination of rivers and streams, and called for some alternative to the FC:FS ratio. Feachem (1975) asserted that initial high FC:FS ratios which fall later, indicate human pollution, whereas initial low values which subsequently rise suggest a non-human faecal pollution. Apart from the obvious drawbacks mentioned in Section 1.7, it is highly improbable and technically impracticable to monitor water sources continuously for 24h and daily for 7 days just to gauge the source of pollution. A review of the indices of pollution is needed.

4.8. Index of Water Quality

Although standards for drinking water are laid down (see p.5 for references), few standards exist for environment water quality. Curiously enough, one would expect an index of pollution for lake, stream or river water quality. A coliform-group D streptococci threshold index is proposed. The application of such an index to water quality would not require a knowledge as to how recent bacterial pollution had taken place. Such an index would point to hazardous pollution of water with which man is likely to come into contact. It has become apparent from the results of this study that a viable coliform count within the range $2.5 - 3.0 \times 10^3$ organisms $100ml^{-1}$, and a viable group D streptococci count within the range $0.88 - 1.0 \times 10^2$ organisms $100ml^{-1}$ signified gross pollution. Such values should always cause some concern and only when counts significantly lower than these values are obtained should such waters be declared safe for human contact.

4.9. Resistance in Coliforms

All of the identified coliform isolates were susceptible to both Cm and Tm. (Table 3.6). Total sensitivity to Cm may reflect the restricted usage of the drug, for on a much larger coliform sample, Linton et al., (1974) found only a 0.2% resistance level. Similar findings for Tm may be due to the fact that until recently it has always been used as a compound drug with sulphamethoxazole. Tc resistance was only detected in E. coli isolates and always occurred as part of a multiple resistance pattern. The low frequency of Tc resistance may suggest that its use as a drug is even more limited in this area than in the Avon area where it represents 2.9% of all prescriptions (Richmond and Linton, 1980). Since most of the Serratia isolates were thought to be Serratia liquefaciens, resistance to Tc in this species was not expected (Siboni,1980). The Citrobacter isolates examined were too few to make any meaningful conclusions.

The frequency of Cx appearing as part of a multiple resistance pattern was higher than that of Tc. The frequency of resistance to Cx may reflect the degree of its usage in the area but information regarding resistance frequency in the literature pertains only to clinical isolates with which no useful comparisons may be made.

The high incidence of Sm resistance was very surprising. Even though this drug is not commonly used, this high incidence cannot be explained with the information available from the location. One can only speculate about the origin of such high incidence of resistance. This drug is produced by soil bacteria and the resistant strains may have come in contact with Sm producers where this selective pressure had favoured their predominance. In addition, it is possible that the resistant strains may have chromosomal genetic information which codes for Sm resistance. The frequency of resistance to Sm was much higher than that reported by Fontaine and Hoadley, (1976) who found a 63.6% frequency of Sm resistance in combined wastes. During this investigation, the MIC level taken as designating resistance to Sm was $4\mu g.ml^{-1}$. This level may be queried but the M.I.C. for E. coli is quoted as 2 Garrod et al., (1973) and also, Lindin-Janson and her co-workers (1977) found 90.6% of E. coli isolates from the rectum of healthy school children had an M.I.C. ≤ 2 for Sm. Authors using higher M.I.C. values have, in general, been working with clinical isolates.

The proportion of isolates resistant to Su among the isolates from below the outfall were comparable with those reported by Grabow and Prozesky, (1973). The relative high number of resistant isolates may be associated with (a) usage and (b) the fact that the sulphonamides have been in regular clinical use since before the second world war.

Among the isolates from below the outfall, the percentage of coliforms resistant to Ap was higher than the 20% quoted by Grabow and Prozesky, (1973) for coliform in city sewage in Pretoria but less than the 48.4%

level in coliforms from domestic sewer in Bristol (Linton et al., 1974). Resistance to Ap alone was not as common as that reported by Linton et al., (1974) and Ap resistance was usually associated with multiple resistance.

The resistance levels in the isolates from the village sampling point may be due to the input from septic tanks or more probably to the use of sewage digestion solids as fertilizer.

The results of the M.I.C. determination(Table 3.6)show that there were significant numbers of drug resistant coliforms from both sampling points. The incidence of resistance was generally more frequent in isolates from below the outfall and this is particularly marked with E. coli. Comparing all of the coliforms from above the outfall with those from below, the increase in resistance below the outfall was statistically significant at the 0.5% level.

4.10. Transferability of Drug Resistance Markers in Coliforms

The percentage of multiple-resistant strains able to transfer resistance to E. coli K-12 (Table 3.9) was higher than that reported by Williams-Smith, (1970) but substantially higher than that reported by Sturtevant and Feary, (1969); Grabow and Prozesky, (1973); Linton et al., (1974) and by Fontaine and Hoadley, (1976). Transfer of five resistance determinants was demonstrated. The resistance determinants for Cx was least transferred and that for Ap most frequently transferred. Since Ap and Cx are structurally similar, one would have supposed that Cx transfer would be as frequent as that of Ap. Three explanations are possible for the non-transfer of Cx resistance. Although unlikely, it would seem possible that Ap and Cx-resistant strains have evolved different mechanisms of resistance. Also, it is possible that there may be more than one plasmid. More likely, is the possibility that Cx resistance may be chromosomal. In general the phenomenon of transferability suggests that the drug resistance markers are plasmid-borne.

4.11. Linked Resistance Transfer in Coliforms

Growth was not detected on combination plates with approximately 90% of the multiple-resistant strains. No useful comparison with the results of other workers could be made in this respect since there appears to be no published reports on the use of combination plates in transfer work. Although evidence for transfer was detected when drugs were added individually, it is surprising that growth was not detected on such combination plates. It is likely that the addition of several antibiotics may be synergistic with the result that the inoculum is sterilized. An examination was made of the individual, dual, triple etc. resistance patterns within the genera in an attempt to postulate the possible order in which resistance genes were acquired by the individual strains. No orderly branched pattern of this type emerged from the results obtained.

4.12. Resistance in Group D Streptococci

Toala et al., (1969) reported on the similarities and differences in the susceptibility of group D streptococci species to various antibiotics. They reported that strains of S. faecalis and its variant zymogenes, were more susceptible to the penicillins, erythromycin and the aminoglycosides, than strains of S. faecium and S. faecalis var. liquefaciens. Also strains of S. faecalis var. liquefaciens were more resistant to the tetracyclines than the others with S. faecium most susceptible to tetracycline. Thornsberry et al., (1974) reported that S. bovis strains were more susceptible to antibiotics than the enterococci. Although the above studies were on human isolates, the M.I.C. results obtained from this investigation from both the environmental and clinical isolates, when compared with the earlier studies appear to be in general agreement, with some exceptions. Apart from the results supporting the conclusions of Thornsberry et al., (1974) that S. bovis is more susceptible to antibiotics than the enterococci, there appears to be no definite pattern

of susceptibility among the various species of the group D streptococci as observed by Toala et al., (1969).

Resistance to Tc was most frequently encountered with 6% of the isolates from above the sewer outfall, 16% from below and 55% of those from clinical sources being resistant. The 16% Tc-resistant isolates from below the outfall is consistent with values of 13% and 15% respectively, quoted by Van Embden et al., (1977) as percentages of Tc-resistant group D streptococci from two sewage plants. The incidence of Tc-resistant strains from clinical sources (55%) differed markedly from that (16%) observed from isolates below the outfall. Values ranging from 40-70% of Tc-resistant group D streptococci from clinical sources have been quoted by other workers (Toala et al., 1969; Van Embden et al., 1977; Marder and Kayser, 1977). Although the percentages of resistant strains from the clinical isolates and among the isolates from below the outfall are not directly comparable because of their differences in origin, the results suggest that Tc-resistant group D streptococci from ostensibly healthy people, is less frequent than that observed in hospitalized people. A probable explanation of this variation may be due to the therapeutic use of the antibiotic. Although some of the streptococcal isolates have been shown to be Tc-resistant, this drug though not the drug of choice in the treatment of streptococcal infection (Parker, 1978; Barrie, 1980), is nevertheless widely used.

With regard to Em resistance, a similar percentage (3%) to that (2.7%) of Van Embden et al., 1977 was observed in isolates from below the outfall. The incidence of Em-resistant strains, although the same criteria were used, among the clinical isolates (6%) was lower than that (57%) of Toala et al., (1969). Whereas no resistance to Gm was observed in isolates from clinical sources, 41.17% of isolates from below the outfall displayed resistance to this drug. Toala et al., (1969) found Gm to be the most active

aminoglycoside against the group D streptococci. There seems to be no obvious explanation for Gm resistance. These Gm-resistant strains were characteristically S. durans. If there were species differences to this drug one would have expected all S. durans strains to be Gm-resistant. The results did not show this to be the case.

Toala et al., (1969) observed that 92.7% of their group D streptococci strains were Sm-resistant. The results from this study show the contrary, i.e. only 23% of the clinical isolates displayed Sm resistance. This variation is almost certainly due to the M.I.C. (μgml^{-1}) level used by Toala et al., (1969) and the level used in this study. Whereas Toala et al., (1969) used a level of > $100\mu gml^{-1}$, the level for Sm used in this study was > $1000\mu gml^{-1}$. This high level may be queried, but the M.I.C. (μgml^{-1}) for S. faecalis is quoted as 64 - 256 (Garrod et al., 1973). Two types of Sm resistance, moderate and high level of resistance, were found amongst the group D streptococci (Table 2.3(b)). Such findings are in agreement with those of Standiford et al., (1970) with wild strains of enterococci.

The mechanism of resistance in the moderately resistant isolates may be due to (i) the impermeability of the antibiotic to the cell, which prevents the drug from reaching the sensitive ribosome target; this permeability barrier is usually overcome by simultaneously exposing such cells to Pn and Sm and may account for the reason why Sm is used in combination in enterococcal endocarditis and (ii) the production of varying levels of induced Sm-inactivating enzymes with modified Sm-binding sites may cause high level of resistance, and simultaneous treatment with Pn is usually without effect (Zimmermann et al., 1971).

In general, the results of the M.I.C. determinations of the group D streptococci from both environmental and clinical sources showed higher

M.I.C. values to Ap and Pn in the clinical strains, but these levels were within the sensitive ranges.

4.13. Multiple Resistance in Group D Streptococci

It has been claimed that S. faecalis is the important pathogenic streptococcus that is resistant to a wide range of antibiotics (Parker, 1978). It is interesting to note that among the 7 multiple-resistant strains, the different species represented were as follows: S. faecalis var. faecalis = 2, S. faecium = 2, S. faecalis var. liquefaciens = 2 and S. faecalis var. zymogenes = 1. This result suggests that multiple-resistance is not restricted to S. faecalis strains and also highlights the need for differentiating the variants of S. faecalis. As far as the distribution of the multiple-resistant strains was concerned, 2 were environmental isolates and 5 from clinical sources. In view of the relatively few multiple-resistant streptococci, there is no basis to support the view that the widespread use of antibiotics has led to the increase in S. faecalis strains resistant to antibiotics. What is disturbing is that this multiple-resistance is transferable via conjugation (Jacob and Hobbs, 1974; Van Embden et al., 1977; Marder and Kayser, 1977; Engel et al., 1980). Whether this phenomenon will lead to increase in multiple-resistance among the group D streptococci remains to be seen. Although Ap and Pn-resistant strains were encountered, it is gratifying to know that majority of the isolates remain susceptible to benzyl penicillin - a therapeutically useful agent.

4.14. Transfer in Group D Streptococci

The frequency of transfer in the streptococci observed in this study was of the same order of magnitude as that reported by Van Embden et al., (1977) on similar studies. Conjugal transfer of resistance factors has been observed in S. faecalis and in S. faecium (Van Embden et al., (1977).

These workers reported that 61% of their streptococci isolates achieved transfer of one or more of their markers to S. faecalis JH 2-2. Furthermore, one of their S. faecium isolates failed to transfer Tc resistance to JH 2-2. The results obtained from this study showed that all of the multiple drug resistant group D streptococci transferred two or more of their markers to JH 2-2. In addition, the two multiple resistant S. faecium strains transferred their resistance markers, except Ap, to the recipient S. faecalis JH 2-2. There appears to be reports on the observation of linked resistance to Ap and Pn in group D streptococci at the time this thesis was prepared. In this study, such resistance was observed and the transferability of Pn only was noted.

It is not easy to explain why Ap resistance was not transferred during conjugation. This may be due to hyper-mutable chromosomal genes. This is being further investigated by other workers in the department.

4.15. Comparison of multiple drug resistance among coliforms and group D streptococci isolates

The incidence of multiple drug resistance appeared to be more frequent among the coliforms than in the group D streptococci. This may be due in part to (a) the inaccessibility of the drugs to the gram-negative cells as a result of their cell wall composition and (b) the possibility of plasmid mediated conjugal transfer within the coliforms. Regardless of the mechanism by which this multiple drug resistance was mediated, these resistant bacteria may have considerable public health significance, since people can be exposed to the stream containing multiple-resistant bacteria. If among such people there are young children, people with impaired immunological responses, debilitated people and people undergoing antibiotic therapy, direct exposure to such contaminated water could present considerable danger to their health.

At first sight it would seem improbably that there are health risks involved considering that these indicator organisms are normally non-

pathogenic. However, it is generally known that these same organisms are often implicated in nosocomial infections and may even be the causative agents of mild diarrhoea. Furthermore, as far as the coliforms are concerned, they seem to be multiple-resistant to the more frequently prescribed drugs which in themselves are less expensive than the new semi-synthetic drugs. This raises the possibility of increase in expenditure in the National Health Service at the time resources are scarce.

Hartley and Richmond, (1975) point out that resistant strains of E. coli in the human gut can have complex long term consequences. Whereas some resistant strains disappear quickly, others persist for months in the absence of obvious antibiotic selection pressures. Such resistant populations may present a potential hazard in the event of human infection as they increase the general level of resistance in the environment (Richmond, 1972; Anderson et al., 1976).

4.16. In vitro stability of plasmids

Curing agents such as acridine orange or ethidium bromide are known to eliminate plasmids from cells that harbour them. They can do so because the organization and conformation of the plasmid is different from those of the chromosome (Gale et al., 1972). It is also probably that they exert their effect by selectively inhibiting plasmid DNA replication (Riva et al., 1973). Curing is achieved by using these agents at sub-inhibitory concentration to bacterial growth whereby plasmid replication is inhibited.

The results obtained from the curing experiments in both groups of bacteria show a very low ability to cure these bacteria of R.-factors. Similar observations for group D streptococci have been made (Clewell et al., 1974; El-Solh et al., 1978). Anderson, (1968) stated that failure to demonstrate instability in the enterobacteriaceae is not evidence against plasmids since many R-plasmids are very stable. It is possible that, at least in the coliforms, the transfer factors may exist in a state of symbiosis with their hosts which has contributed to their stability.

Anderson, (1968) stated that "R-factors are usually more sensitive to acriflavine than acridine orange and that the efficiency in which they are eliminated is generally much lower than is the case of F-factors". Probably the ineffectiveness of acridine orange more than any other possible factor may be responsible for the low rate of curing.

The patterns of spontaneous loss encountered in the coliforms were Ap and Sm, Cx Sm or single loss of Ap or Sm. With the group D streptococci only Ap was spontaneously lost. Spontaneous loss has been attributed to segregation during logarithmic growth resulting from asynchromy between replication of the resistance determinants, the transfer factor and the cell (Anderson, 1968).

4.17. Group D streptococci plasmids

The sizes of the plasmids found were different from those that have been reported (Clewell et al., 1974; Marder and Kayser, 1977). It is possible that the plasmids may belong to the same class as those previously reported. It is also possible that they may represent a totally different class of plasmids. More work needs to be done to characterize these plasmids.

4.18. Survival of Coliforms

Zaske et al., (1980) have shown that the survival of E. coli in a water environment is short-lived. This is because E. coli has its normal habitat in the human or animal intestine, and is used to relatively high temperatures, osmotic pressure and nutrient levels. The results from this study supported this view. Although E. coli is the most widely used indicator organism of faecal pollution (1.6.1) its poor power of survival outside the human or animal body should preclude its use as the sole indicator organism as even the pathogens it is supposed to indicate survive better (Buttiaux and Mossel, 1961).

The other members of the coliform genera, such as Enterobacter, Citrobacter and Klebsiella have been reported to have longer survival periods because they can be found both in the intestine and the environment (see Chapter 1.2). The results also support this view and the evidence is suggestive that except Enterobacter species, they survive longer than E. coli in the natural environment.

Although this initial study supports the views of other workers, the study of the survival of mixtures of pure strain cultures is needed. Information from such studies would help in better understanding of the factors contributing to the differential die-off rates observed in this group.

4.19. Survival of Anaerobes

High counts were recorded for bacteroides and fusobacteria. Sporulating clostridia and anaerobic cocci were also in appreciable numbers. The figures quoted for Bacteroides fragilis and for sporulated clostridia were of the same order as these quoted by Opara, (1978) and by Watkins and Sleath, (1981) for clostridia. No veillonellas, eubacteria or bifidobacteria were presumptively identified although the identity of a number of anaerobic bacteria was unknown. The numbers of sporulated clostridia were low compared with the total of clostridial colonies seen on the selective plates.

The problem associated with an all-purpose medium such as blood agar, for the isolation of anaerobes, is that of differentiating facultative anaerobic bacteria from the obligate anaerobes. Whereas with an all-purpose medium the detection of obligate anaerobes would require the picking off of all colonial types which may be numerous, and subculturing aerobically and anaerobically, the number of colonies developing on a selective medium are greatly reduced. Selective media are to some extent suppressive but much labour and time could be saved by using such media in anaerobic work. Thus, it must be stated that the obligate anaerobic count at the input end of the system was not less than $2.3 \times 10^{6} ml^{-1}$ and not less than $1.6 \times 10^{5} ml^{-1}$ in the final effluent end.

The number of anaerobic bacteria released into the environment raises the question of the additional implication of anaerobes in public helath hazard. Amongst the genera isolated are several pathogens and opportunistic pathogens. Although the occurrence of anaerobic bacteria, with the exception of clostridia in water, has not played a significant part in water bacteriology further studies on their association with the enteric aerobic bacteria is required. It has been suggested that the control of the numbers of intestinal E. coli appears to be predominantly due to anaerobes (Broda, 1979). Further work is required to elucidate this hypothesis in the natural environment.

4.20. Future Prospects

Water quality has been radically transformed in the past sixty years. New methods for the rapid detection of microorganisms responsible for pollution is required. The most urgent need - apart from rapid detection - is for a reliable and versatile medium for the isolation of anaerobic bacteria, and of the characterisation of S. faecium var. casseliflavus.

Many bacterial species, both pathogenic and non-pathogenic, have been known to survive in water. For both species we need to know the physical and chemical conditions under which they adjust to changes and the optimum conditions for their survival and reproduction. Attempts have been made but the picture remains unclear. With such knowledge one could predict whether the introduction of domestic and animal wastes would produce a transient or lasting effect on the environment.

Biologists are well aware of the ability of man to alter the face of the earth. Could continued pollution of the environment be a built-in corrective of population explosion? Could it be true that natural selection in bacteria preserves those that are useful and rejects those that are of negative value? The rapid development of antibiotic resistance in bacteria has provided an almost paradigm of natural selection, giving the microbiologist and the molecular biologist a chance to observe major evolutionary changes in a few years. It seems that when the conditions are right, evolution needs not be a slow progress. The widespread use of antibiotic kills the normally susceptible bacteria leaving a tiny minority that have resistance factors to survive. When the conditions are right, those resistant cells may multiply in the natural environment both because they have a common origin and are in a restricted area, and because each reacts to the environmental factors in much the same way. This may make conjugation much easier to achieve by ensuring physical

contact and by enhancing the possibility for conjugal transfer and thereby for the evolution of the resistant species.

Physicians are uneasily aware that infectious diseases have not been conquered but are kept at bay at a given population density. If the density becomes high enough and the level of pollution of the environment increases, some of the disease-producing organisms which have devastated mankind throughout history can be expected to return. What is frightening is that they may return armed with resistance factors. What this could mean is that man's survival on the earth's surface would be more difficult.

In the foregoing section, the future of man in relation to his environment has been painted. If the picture remains incomplete and less than satisfying it should be noted that there are still major discoveries to be made. What these discoveries may be, there is no clear idea and they may be made because man has always addressed himself to interesting challenges.

5. APPENDICES

APPENDIX A

The undermentioned publication, based on material included in this thesis, has already been made.

D. THIRKELL and M. BIANKSON

The speciation of coliform genera from above and below a sewer outfall and their susceptibilities to antimicrobial agents.

Antonie van Leeuwenhoek 47, (1981) 133-145.

APPENDIX B

Oxoid Membrane Enriched Teepol Broth (METB) contained:

Bacteriological Peptone grams per litre	40.0
Yeast extract	6.0
Lactose	30.0
Phenol red	0.2

pH 7.4 (approximately)

add 4ml of Teepol 610 (B.D.H. Ltd.)

Oxoid Slanetz and Bartley Medium contained:

Tryptose	grams per litre	20.0
Yeast extract		5.0
Dextrose		2.0
Disodium phosphate. $2H_2O$		4.0
Sodium azide		0.4
Tetrazolium chloride		0.1
Agar No.1		10.0

pH 7.4 (approximately)

TABLE TABLE FOR THE DIFFERENTIATION OF THE ENTEROBACTERIA AFTER COWAN (1974)

	Citrobacter spp.	Enterobacter spp.	Klebsiella spp.	Serratia spp.
Catalase	+	+	+	+
Gluconate	N/D	+	+/ - /d	+
Malonate	+/-	d/+	+/ - /d	-/d
Urease	-/w	d	+/ - /d	-/w/d
H_2S	+/-	-	-	-
Acid from inositol	N/D	-/+	+/d	+/d
Gas from glucose	+	+	+/ - /d	+/d
ONPG	+	+	+	+
MR	+	-	-/ + /d	+
VP	-	+	+/ - /d	d
Indole	+/-	-	-/+	-
Gelatin	-	d/(+)	-/+	+
Phenylalanine	-	-	-	-
Citrate	+	+	+/ - /d	+/d
Oxidase	-	-	-	-
Motility	+	+	-	+
DNase	-	-	-	+

(+) = 85-100% strains are positive (all, most, many, usually)
- = 0-15% strains positive (no, none, few, some)
d = 16-84% strains positive (many, some)
w = Reaction delayed and weak
N/D = No data

TABLE TABLE FOR THE DIFFERENTIATION OF THE GROUP D STREPTOCOCCI AFTER FACKIAM, 1972; COWAN, 1974; GROSS et al. 1975; Parker and Ball, 1976.

	S. faecalis var. faecalis	S. faecalis var. liquefaciens	S. faecalis var. zymogenes	S. faecium	S. faecium var. casselflavus	S. durans	S. bovis	S. equinus
Growth at 10°C	+	+	+	+	+	+	-	-
Growth at 45°C	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	-	-
Growth on 10% BAeM	+	+	+	+	+	+	+	+
Growth on 40% BAeM	+	+	+	+	+	+	+	+
0.04% tellurite tolerance	+	+	+	-	-	-	-	-
Reduction of 0.1% tetrazolium	+	+	+	-	-	-	-/+	-
Mead's Medium	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Hydrolysis of starch	-	-	-	-	-	-	+/-	+/-
Liquefaction of gelatin	-	+	-	-	-	-	-	-
Pigment production	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Apparent pigment production	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Acid from: Mannitol	+	+	+	+	+	-/+	+	+
Glycerol(AN)	+	+	+	-	-	-	-	-
Arabinose	-	-	-	+	+/-	-	-	-
Melezitose	+/-	+/-	+/-	-	-	-	-	-
Sorbitol	+	+	+	-	-	-	-	-
Inulin	-	-	-	-	+/-	-	-	-
Melibiose	-	-	-	+	-/+	-/+	+/-	-
Lactose	+	+	+	+	+	+	+	-/+
Hydrolysis of arginine	+	+	+	-	-	-	-	-
Fermentation of pyruvate	+	+	+	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-

\+ = Positive result

\- = Negative result

N/D = No data

APPENDIX 1 (a) CHARACTERIZATION TESTS FOR THE IDENTIFICATION OF COLIFORMS FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Catalase	Gluconate	Malonate	Urease	H_2S	Acid from inositol	Gas from glucose	ONPG	MR Test	VP Test	Indole	Gelatin hydrolysis	Phenylalanine	Citrate as C source	Oxidase	Motility	DNase	Tween 80	Identification
CA56	+	−	+	−	+	+	+	+	+	−	−	−	−	+	−	+	−	−	Citrobacter spp
63	+	−	+	−	−	+	+	+	+	−	−	−	−	+	−	+	−	−	"
CA11	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	Enterobacter spp.
15	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
16	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
18	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
20	+	+	+	+	−	−	+	+	−	+	−	+	−	+	−	+	−	−	"
22	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
27	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
40	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
41	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
42	+	−	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
58	+	−	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
59	+	+	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	−	"
61	+	+	+	−	−	+	+	+	−	+	−	−	−	+	−	+	−	−	"
64	+	+	−	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
68	+	−	+	−	−	+	+	+	−	+	−	−	−	+	−	+	−	−	"
69	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
72	+	+	+	−	−	+	+	+	−	+	−	−	−	+	−	+	−	−	"
73	+	+	+	−	−	+	+	+	−	+	−	−	−	+	−	+	−	−	"
74	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
77	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"
78	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	+	−	−	"

+ positive result − negative result

APPENDIX 1 (a) Contd. CHARACTERIZATION TESTS FOR THE IDENTIFICATION OF COLIFORMS FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Catalase	Gluconate	Malonate	Urease	H_2S	Acid from inositol	Gas from glucose	ONPG	MR Test	VP Test	Indole	Gelatin hydrolysis	Phenylalanine	Citrate as C source	Oxidase	Motility	DNase	Tween 80	Identification
CA79	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	Enterobacter spp.
80	+	+	+	-	-	-	+	+	-	+	+	-	-	+	-	+	-	-	"
84	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
87	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
91	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
95	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
96	+	+	+	-	-	-	+	+	-	+	-	+	-	+	-	+	-	-	"
97	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
99	+	+	+	-	-	-	+	+	-	+	-	+	-	+	-	+	-	-	"
13	+	+	-	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	Klebsiella spp.
23	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
25	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
32	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
44	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
49	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
53	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-	"
70	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
71	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
81	+	+	-	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
88	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	Serratia spp.
89	+	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	+	+	"
94	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	"

+ positive result - negative result

APPENDIX 1 (b) CHARACTERIZATION TESTS FOR THE IDENTIFICATION OF COLIFORMS FROM BELOW THE SEWER OUTFALL

Catalogue Number	Catalase	Gluconate	Malonate	Urease	H_2S	Acid from inositol	Gas from glucose	ONPG	MR Test	VP Test	Indole	Gelatin hydrolysis	Phenylalanine	Citrate as C source	Oxidase	Motility	DNase	Tween 80	
CB 7	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+	-	-	Enterobacter spp.
10	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
19	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	+	-	-	"
41	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
44	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	+	-	-	"
47	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
48	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
53	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
57	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	+	-	-	"
66	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
69	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
71	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
72	+	+	-	-	-	+	+	+	-	+	-	-	-	+	-	+	-	-	"
81	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
83	+	+	-	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
86	+	+	-	-	-	+	+	+	-	+	-	-	-	+	-	+	-	-	"
94	+	+	-	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	"
1	+	-	-	-	-	-	+	+	+	-	+	-	-	+	-	+	-	-	Citrobacter spp.
59	+	-	+	-	-	+	+	+	+	-	-	-	-	+	-	+	-	-	"
95	+	-	-	-	-	-	+	+	+	-	-	-	-	+	-	+	-	-	"

+ positive result - negative result

APPENDIX 1 (b) (Contd.) CHARACTERIZATION TESTS FOR THE IDENTIFICATION OF COLIFORMS FROM BELOW THE SEWER OUTFALL

Catalogue Number	Catalase	Gluconate	Malonate	Urease	H_2S	Acid from inositol	Gas from glucose	ONPG	MR Test	VP Test	Indole	Gelatin hydrolysis	Phenylalanine	Citrate as C source	Oxidase	Motility	DNase	Tween 80	
CB 2	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	Klebsiella spp.
21	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
30	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
35	+	+	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	-	"
36	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-	"
40	+	-	+	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-	"
42	+	+	-	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
58	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
63	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
65	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
68	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
76	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
84	+	+	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	-	"
85	+	+	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	-	"
87	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
91	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	"
20	+	-	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	Serratia spp.
23	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+		"
26	+	+	-	-	-	+	+	+	+	-	-	+	-	+	-	+	+		"
32	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	+	+	"
45	+	+	-	-	-	+	+	+	+	-	-	+	-	+	-	+	+	+	"
62	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	"
96	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	"
99	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	"
100	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	"

+ positive result - negative result

APPENDIX 2 (a) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEL	Growth on 40% BEL	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Iannitol	Glycerol (AH)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
Sx1	-	+	-	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	+	+	-	-	S. durans
2	-	-	+	+	-	+	-	+	+	+	+	-	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-	Unidentified
3	+	-	-	+	+	+	+	+	+	-	-	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	-	S. faecium
4	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	++	+	-	S. faecalis var. zymogenes
5	-	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-	-	Unidentified
6	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	-	-	-	+	+	-	-	S. faecium
7	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+	-	-	S. faecalis var. casseliflavus
8	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	S. durans
9	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	+	-	+	-	+	+	-	-	S. faecium
10	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	+	-	-	+	+	+	-	S. faecalis var. faecalis
11	+	-	-	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	-	-	-	-	+	+	-	-	S. faecium var. casseliflavus
12	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	+	-	+	+	-	-	S. faecium
13	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
14	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	+	-	-	S. faecium
15	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	+	+	+	-	+	-	-	+	+	-	-	S. faecium var. casseliflavus
16	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
17	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	+	+	-	-	S. faecium var. casseliflavus

\+ positive result

\- negative result

APPENDIX 2 (a) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BE.	Growth on 40% BE.	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Lannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SA18	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	+	-	-	S. faecium
19	-	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
20	-	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	S. durans
21	-	-	-	+	+	+	+	+	+	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+	+	-	-	S. faecium
22	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	-	S. faecium var. casseliflavus
23	-	-	-	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	+	-	+	-	+	+	+	-	-	S. faecium var. casseliflavus
24	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	+	-	-	S. faecium
25	-	-	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
26	-	-	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	-	-	-	+	+	+	-	Sa. faecalis var. liquefaciens
27	-	-	+	+	+	+	+	-	+	-	-	+	-	-	-	-	+	+	+	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
28	-	-	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	+	+	-	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
29	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	+	-	-	+	+	+	-	S. faecium var. casseliflavus
30	-	-	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	-	+	+	+	-	"
31	-	-	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	-	+	+	+	-	"
32	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	-	-	+	+	+	-	"
33	-	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	-	S. faecium
34	-	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	+	+	-	+	-	-	+	+	+	-	S. faecium var. casseliflavus

\+ positive result

\- negative result

APPENDIX 2 (a) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEL	Growth on 40% BEL	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Lamnitol	Glycerol (AM)	Arabinose	Lelezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SA35	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	-	-	+	+	+	-	-	S. faecium var. casseliflavus
36	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	-	+	+	+	-	-	S. faecium
37	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	+	+	+	-	S. faecalis var. faecalis
38	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	+	-	+	+	+	-	-	S. faecium
39	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	"
40	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	+	-	-	+	+	-	-	S. faecium var. casseliflavus
41	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	-	-	-	+	+	-	-	"
42	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	+	-	-	S. faecium
43	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	S. faecium var. casseliflavus
44	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	"
45	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	+	-	+	+	+	-	-	S. faecium
46	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	+	-	+	+	+	-	-	S. faecalis var. casseliflavus
47	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	+	-	+	-	+	+	+	-	-	"
48	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	-	S. faecalis var. faecalis
49	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	S. durans
50	-	-	+	+	-	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	S. bovis
51	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	-	+	+	+	+	-	-	S. faecium var. casseliflavus

+ positive result

- negative result

APPENDIX 2 (a) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Iannitol	Glycerol (AM)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SA52	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	S. durans
53	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	"
54	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	+	+	+	+	−	−	S. faecium
55	−	+	−	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	−	−	S. durans
56	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	−	S. faecium var. casseliflavus
57	−	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	−	−	−	+	+	+	+	−	S. faecalis var. faecalis
58	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	"
59	−	−	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	−	−	+	+	+	−	−	S. faecium var. casseliflavus
60	−	−	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	−	−	+	+	+	−	−	"
61	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	+	−	+	+	+	+	+	−	−	"
62	−	−	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	−	−	+	+	+	−	−	"
63	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	−	S. durans
64	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	+	−	+	+	+	+	−	S. faecalis var. faecalis
65	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	−	S. durans
66	−	−	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+	−	+	−	−	−	+	+	+	−	−	S. faecium var. casseliflavus
67	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	S. durans
68	−	−	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	−	+	+	+	+	−	−	S. faecium var. casseliflavus

\+ positive result

\- negative result

APPENDIX 2 (a) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Meads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AM)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SA69	-	+	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	-	-	-	-	-	+	+	-	-	S. durans
70	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	+	+	-	-	S. faecium
71	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	-	+	+	-	-	"
72	-	-	+	+	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	-	-	-	+	+	+	-	S. faecium var. casseliflavus
73	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	+	+	-	-	S. faecium
74	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	+	+	-	-	"
75	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	+	+	-	S. faecium var. casseliflavus
76	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	-	+	+	+	-	S. faecium
77	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-	"
78	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	+	+	+	+	-	"
79	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	-	+	+	+	+	S. faecalis var. faecalis
80	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	-	+	+	+	-	S. faecium var. casseliflavus
81	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	+	+	-	"
82	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+	+	-	-	S. faecium
83	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	-	+	+	-	-	S. faecium var. casseliflavus
84	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	-	+	+	+	+	-	-	S. faecium
85	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	-	+	+	-	-	"

\+ positive result

\- negative result

APPENDIX 2(a) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM ABOVE THE SEWER OUTFALL

Catalogue Number	Haemolysis			Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEN	Growth on 40% BEN	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
	α	β	γ																								
SA86	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
87	-	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	+	-	-	-	+	+	+	-	-	S. faecium var. casseliflavus
88	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	-	S. faecium
89	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
90	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	-	S. faecium var. casseliflavus
91	-	+	-	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	S. durans
92	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	S. faecalis var. faecalis
93	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecium var. casseliflavus
94	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	-	-	+	+	-	-	S. faecium
95	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	S. faecalis var. faecalis
96	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	"
97	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	S. bovis
98	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	S. faecalis var. faecalis
99	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	S. faecium
100	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	S. faecium var. casseliflavus

\+ positive result

\- negative result

APPENDIX 2 (b) CHARACTERISATION TEST FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM BELOW THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Meads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SB 1	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	S. durans
2	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	S. faecium
3	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	"
4	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	"
5	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	"
6	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	"
7	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	S. durans
8	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	"
9	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	"
10	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	"
11	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	−	+	+	+	+	−	S. faecium
12	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	+	+	+	−	S. durans
13	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	S. faecalis var. faecalis
14	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	"
15	−	−	+	+	+	+	+	+	+	−	−	−	+	−	−	−	+	+	+	+	+	−	−	+	+	+	−	S. faecium
16	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	−	+	+	+	−	+	+	+	+	−	S. faecalis var. faecalis
17	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	−	+	+	+	−	−	+	+	+	−	"

\+ positive result

\- negative result

APPENDIX 2 (b) (Contd.) CHARACTERISATION TEST FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM BELOW THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Neads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Lamitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SB13	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	-	-	S. faecalis var. faecalis
19	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	-	-	"
20	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	"
21	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	S. faecium
22	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	-	-	S. faecalis var. faecalis
23	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	"
24	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	-	-	Unidentified
25	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	S. faecalis var. faecalis
26	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	-	-	S. durans
27	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	S. faecalis var. faecalis
28	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	"
29	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	-	-	S. durans
30	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	-	-	S. faecium
31	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	S. faecium var. casseliflavus
32	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	S. faecalis var. faecalis
33	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	-	-	S. faecium
34	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	-	-	S. faecium var. casseliflavus

\+ positive result

\- negative result

APPENDIX 2 (b) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM BELOW THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Meads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SB35	-	ı	+	+	+	+	+	+	+	+	+	+	ı	ı	ı	ı	+	+	-	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
36	-	-	+	+	+	+	+	+	+	ı	ı	ı	ı	ı	ı	ı	+	ı	+	ı	+	-	+	+	+	-	-	S. faecium
37	-	+	ı	+	+	+	+	+	+	+	+	+	ı	ı	ı	+	+	+	+	+	+	-	+	+	+	-	-	S. faecalis var. zymogenes
38	-	ı	+	+	+	+	+	+	+	+	+	+	ı	ı	ı	ı	+	-	+	-	+	-	-	+	+	+	-	S. faecalis var. faecalis
39	-	-	+	+	+	+	+	+	+	+	+	+	ı	ı	ı	ı	+	+	+	-	+	-	-	+	+	+	-	"
40	-	-	+	+	+	+	+	+	+	+	+	+	ı	-	ı	+	+	+	+	-	+	-	-	+	+	+	-	"
41	+	-	ı	+	+	+	+	+	+	+	+	ı	ı	-	+	-	+	-	+	-	-	-	+	+	+	-	-	S. faecium var. casseliflavus
42	+	ı	-	+	+	+	ı	+	+	+	+	ı	ı	ı	+	-	+	-	+	-	-	-	-	+	+	-	-	"
43	ı	-	+	+	-	+	ı	+	+	+	ı	ı	+	ı	ı	ı	+	+	+	+	-	+	+	+	+	-	-	S. bovis
44	ı	-	+	+	+	+	+	+	+	+	+	+	ı	ı	ı	+	+	+	+	-	+	-	-	+	+	+	-	S. faecalis var. faecalis
45	-	-	+	+	+	+	+	+	+	+	+	+	-	-	ı	ı	+	+	+	-	+	-	-	+	+	+	-	S. faecium
46	-	-	+	+	+	+	+	+	+	+	+	+	ı	ı	ı	+	+	+	+	-	+	-	-	+	+	+	-	S. faecalis var. faecalis
47	-	-	+	+	+	+	+	+	+	+	+	+	ı	ı	ı	+	+	+	+	-	+	-	-	+	+	+	-	"
48	-	-	+	+	+	+	+	+	+	ı	ı	ı	ı	ı	ı	ı	+	+	+	ı	+	-	-	+	+	+	-	S. faecium
49	+	ı	+	+	+	+	+	+	+	+	ı	ı	ı	-	ı	ı	+	+	+	ı	+	-	-	+	+	+	-	Unidentified
50	-	+	-	+	+	+	+	+	+	+	ı	ı	ı	-	ı	ı	+	-	+	ı	+	-	-	+	+	+	-	S. durans
51	-	-	+	+	+	+	+	+	+	ı	ı	ı	ı	-	ı	ı	+	+	+	ı	+	-	-	+	+	+	-	S. faecium

\+ positive result

\- negative result

APPENDIX 2(b) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM BELOW THE SEWER OUTFALL

Catalogue Number	Haemolysis: α	Haemolysis: β	Haemolysis: γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Meads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SB52	−	+	−	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	+	+	+	−	S. faecalis var. zymogenes
53	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	+	+	+	−	S. faecalis var. faecalis
54	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	S. durans
55	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	S. faecium
56	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	+	+	+	−	S. faecalis var. faecalis
57	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	S. durans
58	+	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	+	−	−	S. faecium
59	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	S. durans
60	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	S. faecium
61	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	S. durans
62	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	S. faecium
63	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	−	"
64	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	−	"
65	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	−	"
66	+	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	−	+	+	+	−	−	Unidentified
67	−	−	+	+	−	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	S. equinus
68	+	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	S. faecium

\+ positive result

\− negative result

APPENDIX 2 (b) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM BELOW THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AH)	Arabinose	Xylezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
SB69	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	-	-	S. faecium
70	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	S. durans
71	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+	-	-	S. faecalis var. faecalis
72	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	S. faecium
73	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	+	+	+	-	-	S. faecalis var. faecalis
74	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	-	+	+	+	-	"
75	-	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	+	-	-	-	-	+	+	-	-	S. faecium var. casseliflavus
76	-	-	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
77	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	-	-	-	+	+	+	-	"
78	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	-	-	+	+	+	-	S. faecalis var. liquefaciens
79	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
80	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
81	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
82	+	-	-	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-	+	+	+	-	-	S. faecium var. casseliflavus
83	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	-	S. faecalis var. faecalis
84	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-	-	+	+	+	+	-	"
85	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-	-	+	+	+	+	-	"

\+ positive result

\- negative result

APPENDIX 2 (b) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM BELOW THE SEWER OUTFALL

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Lamnitol	Glycerol (AH)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
S386	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	+	+	+	-	S. faecalis var. faecalis
87	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	S. durans
88	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	S. faecium
89	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	S. durans
90	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	-	-	+	+	-	+	+	+	-	-	S. faecalis var. liquefaciens
91	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	-	-	-	S. faecium
92	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	+	+	+	-	-	S. faecium var. casseliflavus
93	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	-	+	+	+	-	-	S. faecalis var. liquefaciens
94	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	+	+	+	-	-	S. faecalis var. faecalis
95	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	S. durans
96	-	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	S. bovis
97	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+	+	-	-	S. faecalis var. faecalis
98	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	+	-	+	+	+	-	-	"
99	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	"
100	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	/	+	-	-	-	+	+	+	+	-	"

+ positive result

- negative result

APPENDIX 2 (c) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
K 1	-	'	+	+	+	+	+	+	+	+	+	+	'	-	-	+	+	-	-	+	+	~	-	+	+	+	-	S. faecalis var. faecalis
2	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
3	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
4	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	-	"
5	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
6	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
7	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes
8	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
9	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
10	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
11	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
12	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
13	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes
14	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
15	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
16	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
17	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis

+ positive result

- negative result

APPENDIX 2 (c) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEL	Growth on 40% BEL	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AK)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
K18	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. zymogenes
19	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	"
20	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	"
21	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	~	S. faecalis var. faecalis
22	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	"
23	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+	-	-	S. faecium var. casseliflavus
24	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. faecalis
25	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	"
26	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. faecalis
27	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. zymogenes
28	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. faecalis
29	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	"
30	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	-	-	"
31	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. liquefaciens
32	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. faecalis
33	-	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	S. durans
34	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	S. faecalis var. faecalis

\+ positive result

\- negative result

APPENDIX 2 (c) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AK)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
K35	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes
36	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
37	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
38	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
39	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
40	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
41	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
42	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
43	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
44	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
45	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
46	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	-	+	-	S. faecium
47	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
48	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	"
49	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes
50	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
51	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	S. durans

\+ positive result

\- negative result

APPENDIX 2 (c) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AN)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
K52	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
53	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	-	"
54	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	-	+	+	-	+	-	S. faecium
55	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
56	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
57	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	"
58	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	Unidentified group D Streptococcus
59	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
60	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
61	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	"
62	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
63	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
64	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
65	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
66	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
67	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
68	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes

\+ positive result

\- negative result

APPENDIX 2 (c) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AR)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
K69	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
70	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
71	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
72	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
73	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
74	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
75	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes
76	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
77	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
78	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. liquefaciens
79	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
80	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. zymogenes
81	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	S. faecalis var. faecalis
82	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
83	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
84	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	-	"
85	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	+	-	-	+	+	+	-	"

\+ positive result

\- negative result

APPENDIX 2 (c) (Contd.) CHARACTERISATION TESTS FOR THE SPECIATION OF GROUP D STREPTOCOCCI FROM CLINICAL SOURCES

Catalogue Number	Haemolysis α	Haemolysis β	Haemolysis γ	Group D reaction	Growth at 10°C	Growth at 45°C	Growth at 6.5% NaCl	Growth on 10% BEM	Growth on 40% BEM	0.04% tellurite tolerance	Reduction of 0.1% tetrazolium	Reduction of tetrazolium and decarboxylation of tyrosine (Heads)	Hydrolysis of Starch	Liquefaction of gelatin	Pigment production	Apparent pigment production	Acid from: Mannitol	Glycerol (AK)	Arabinose	Melezitose	Sorbitol	Inulin	Melibiose	Lactose	Hydrolysis of Arginine	Fermentation of pyruvate	Catalase	Species identification
X86	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. liquefaciens
87	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. zymogenes
88	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. liquefaciens
89	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
90	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. liquefaciens
[illegible]	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
92	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. liquefaciens
93	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
94	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	"
25	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	S. durans
96	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
97	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	"
98	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. zymogenes
99	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. faecalis
100	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	+	+	-	S. faecalis var. zymogenes

\+ positive result

\- negative result

APPENDIX 3. (a) M.I.C. LEVELS OF COLIFORM ISOLATES FROM ABOVE THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Sm	Tc	Su	Cx	Cm	Tm
CA 1		4	16	2	>64	16	4	< .18
2		8	16	2	2	8	8	< .18
3		4	16	16	8	8	8	< .18
4		8	8	16	8	8	8	< .18
5		4	8	2	>64	64	4	1.5
6		8	8	4	4	8	8	< .18
7		8	4	.125	8	32	4	< .18
8		4	16	2	16	8	8	.375
9	E. coli	4	8	1	32	8	4	.75
10		16	4	.5	1	4	1	< .18
12		8	8	2	16	4	8	.375
14		8	8	2	1	8	4	< .18
17		4	16	4	4	8	8	< .18
19		8	16	2	2	8	4	< .18
21		8	16	2	2	8	8	.375
24		8	4	1	1	4	2	< .18
26		4	16	2	1	8	4	< .18
28		8	8	2	8	8	8	< .18
29		4	8	2	1	8	8	< .18
30		8	8	2	8	8	8	< .18
31		16	16	2	32	16	8	< .18

Cat.No.	Isolate	M.I.C. in μgml^{-1} Ap	Sm	Tc	Su	Cx	Cm	Tm
CA33		8	16	2	32	8	4	< .18
34		4	16	2	32	16	4	< .18
35		8	8	2	32	16	4	.375
36		8	8	2	2	8	4	< .18
37		4	16	2	2	8	4	< .18
38		4	16	2	32	8	4	< .18
39		4	16	2	64	8	4	.375
43		>64	16	>64	>64	16	4	< .18
45	E. coli	4	8	2	1	8	4	< .18
46		4	8	1	2	8	4	< .18
47		8	16	1	1	8	4	< .18
48		4	8	2	2	8	4	< .18
50		4	16	1	>64	8	4	.375
51		4	8	1	2	8	4	< .18
52		4	8	1	2	4	4	< .18
54		8	>64	>64	4	16	4	< .18
55		4	16	2	8	8	4	< .18
57		8	16	1	4	8	4	< .18
60		4	4	2	8	8	4	< .18
62		4	16	2	2	8	4	< .18
65		4	16	2	2	8	4	< .18

APPENDIX 3. (a) (Contd.) M.I.C. LEVELS OF COLIFORM ISOLATES FROM ABOVE THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Sm	Tc	Su	Cx	Cm	Tm
CA66	E. coli	4	16	2	2	8	4	< .18
67		>64	16	>64	>64	16	4	< .18
75		8	8	2	2	16	4	< .18
76		8	8	2	1	4	4	< .18
82		4	16	2	4	8	4	< .18
83		4	8	2	2	8	4	< .18
85		4	16	2	2	4	8	< .18
86		4	16	2	4	8	4	< .18
90		8	16	2	1	4	4	< .18
92		4	16	2	4	8	8	< .18
93		>64	16	>64	>64	16	4	< .18
98		2	16	2	2	4	4	< .18
100		4	8	2	2	4	4	< .18
56	Citrobacter spp.	16	4	2	>64	8	4	< .18
63		16	2	4	8	16	2	< .18
11	Enterobacter spp.	16	1	2	2	4	1	< .18
15		16	1	1	>64	32	4	< .18
16		8	4	1	2	2	1	< .18
18		16	1	2	8	32	4	.375
20		16	2	2	8	4	4	.375
22		32	4	1	>64	32	2	< .18

Cat.No.	Isolate	M.I.C. in μgml^{-1} Ap	Sm	Tc	Su	Cx	Cm	Tm
CA27	Enterobacter spp.	4	4	1	1	32	2	< .18
40		16	1	2	32	16	4	.375
41		32	2	1	16	>32	1	< .18
42		8	8	2	2	16	4	< .18
58		4	4	1	1	8	2	< .18
59		16	1	2	8	8	2	.375
61		4	4	1	1	16	2	< .18
64		4	2	2	8	>32	4	< .18
68		16	2	2	8	2	1	< .18
69		8	1	4	8	8	2	< .18
72		16	1	4	2	8	4	< .18
73		16	1	2	1	4	4	< .18
74		16	1	8	1	8	4	< .18
77		8	4	1	1	4	4	< .18
78		4	4	1	1	4	2	< .18
79		16	1	4	8	16	4	< .18
80		4	4	1	1	16	1	< .18
84		8	4	2	8	4	1	.75
87		16	4	2	>64	8	2	.375
91		1	2	2	1	4	2	< .18
95		16	4	1	1	4	2	< .18

APPENDIX 3. (a) (Contd.) M.I.C. LEVELS OF COLIFORM ISOLATES FROM ABOVE THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Sm	Tc	Su	Cx	Cm	Tm
CA96		16	4	1	2	4	2	.375
97	Enterobacter	1	4	2	32	8	2	.375
99	spp.	8	1	4	>64	8	4	<.18
13		32	4	1	2	4	2	<.18
23		16	4	1	1	4	2	<.18
25		16	4	1	2	4	4	<.18
32	Klebsiella	16	8	1	4	4	4	<.18
44	spp.	>64	4	1	32	4	4	<.18
49		16	4	1	2	4	2	<.18
53		8	4	1	2	4	2	<.18
70		32	1	1	32	4	1	.375
71		16	1	4	8	4	4	.375
81		32	4	1	32	4	2	.375
88	Serratia	16	1	2	32	8	4	<.18
89	spp.	16	1	1	8	4	2	<.18
94		16	1	2	32	8	4	<.18

Cat.No.	Isolate	M.I.C. in μgml^{-1} Ap	Sm	Tc	Su	Cx	Cm	Tm

APPENDIX 3.1 (b) M.I.C. LEVELS OF COLIFORM ISOLATES FROM BELOW THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in $\mu g ml^{-1}$ of: Ap	Sm	Tc	Su	Cx	Cm	Tm
CB 3		> 32	16	1	>64	>64	2	1.5
4		4	4	2	>64	8	8	< .18
5		1	2	1	4	1	2	< .18
6		4	16	2	>64	16	4	< .18
8		> 32	8	1	4	4	2	< .18
9		8	8	1	16	16	8	.375
11		>32	4	1	4	4	2	< .18
12		4	4	4	1	8	8	< .18
13	E. coli	32	4	2	32	4	2	.75
14		> 32	8	1	32	>64	8	< .18
16		4	4	2	16	4	4	< .18
17		32	8	1	32	8	2	.375
18		>32	4	2	4	>32	8	< .18
22		32	4	1	1	4	2	< .18
24		32	4	1	64	4	2	< .18
25		4	>64	>64	>64	8	8	< .18
27		4	>64	>64	>64	8	8	< .18
28		> 32	>64	2	>64	16	2	< .18
29		32	4	1	1	4	2	< .18
31		8	16	2	64	64	4	< .18
33		32	4	1	2	4	2	< .18

Cat.No.	Isolate	M.I.C. in $\mu g ml^{-1}$ Ap	Sm	Tc	Su	Cx	Cm	Tm
CB34		4	>64	>64	>64	8	4	< .18
37		1	4	1	2	4	2	< .18
38		8	>64	>64	>64	8	8	< .18
39		>32	4	1	2	4	2	< .18
43		8	>64	>64	>64	8	8	.375
46		4	8	2	8	4	8	< .18
49		4	>64	>64	>64	8	8	< .18
50		8	4	2	16	16	1	.375
51	E. coli	8	4	8	64	16	1	.375
52		>32	4	1	4	4	4	< .18
54		4	8	2	2	4	4	< .18
55		4	8	2	1	8	4	< .18
56		4	8	2	4	16	4	< .18
60		4	8	1	4	8	4	< .18
61		> 32	8	4	> 64	> 64	8	.375
64		32	4	1	2	4	4	< .18
70		4	4	2	> 64	4	4	.375
73		8	16	2	4	8	8	< .18
74		8	>64	>64	>64	8	8	< .18
75		8	8	2	4	8	8	< .18
77		8	8	1	32	32	1	< .18

APPENDIX 3.[illegible], (b) M.I.C. LEVELS OF COLIFORM ISOLATES FROM BELOW THE SEWER OUTFALL (Contd.)

Cat.No.	Isolate	M.I.C. in $\mu g ml^{-1}$ of: Ap	Sm	Tc	Su	Cx	Cm	Tm
CB78		2	16	2	>64	8	4	< .18
79		2	2	1	8	16	1	< .18
80		16	4	1	32	4	1	< .18
82		>32	4	1	2	8	8	< .18
88	E. coli	>32	16	1	16	>64	8	.375
89		>32	16	1	16	>64	8	< .18
90		8	8	1	64	4	4	< .18
92		4	16	2	4	4	4	< .18
93		8	16	64	64	16	8	< .18
97		32	16	2	64	64	8	.375
98		2	16	2	1	4	4	< .18
1	Citrobacter	32	8	2	64	8	8	.375
59	spp.	8	4	1	64	8	4	.375
95		8	4	1	8	4	4	.375
7		32	4	1	8	8	2	.375
10	Enterobacter	32	4	1	8	4	2	.75
19	spp.	32	4	4	1	4	2	< .18
41		16	4	2	4	4	4	.375
44		32	4	1	1	4	2	< .18
47		8	4	1	4	4	2	< .18
48		32	4	1	4	4	2	< .18

Cat.No.	Isolate	M.I.C. in $\mu g ml^{-1}$ Ap	Sm	Tc	Su	Cx	Cm	Tm
CB53		32	4	1	1	4	2	< .18
57		>32	4	4	32	16	8	.375
66		8	4	1	2	4	4	< .18
69		16	4	1	2	4	2	< .18
71	Enterobacter	8	2	1	1	4	4	< .18
72	spp.	8	4	1	8	16	4	< .18
81		8	8	2	64	32	8	.375
83		8	8	2	2	16	8	< .18
86		2	4	2	4	8	4	< .18
94		32	4	1	1	32	4	< .18
2		>32	4	4	2	8	4	< .18
21		32	4	1	1	4	2	< .18
30		>32	4	1	2	4	2	< .18
35	Klebsiella	32	4	2	64	4	4	< .18
36	spp.	8	8	1	1	4	2	< .18
40		8	4	1	8	2	2	< .18
42		8	4	1	1	2	2	< .18
58		> 32	4	2	2	8	4	.375
63		32	8	2	16	4	4	.375
65		32	4	1	4	4	4	< .18
68		32	4	1	2	4	2	< .18

APPENDIX 3.3 (b) (Contd.) M.I.C. LEVELS OF COLIFORM ISOLATES FROM BELOW THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in $\mu g ml^{-1}$ of: Ap	Sm	Tc	Su	Cx	C	Tm
CB76	Klebsiella spp.	8	4	1	1	4	2	< .18
84		>32	8	1	16	4	8	< .18
85		32	8	1	16	4	4	< .18
87		16	4	1	4	4	8	.375
91		32	2	1	1	4	2	< .18
20	Serratia spp.	1	2	1	1	2	1	< .18
23		1	1	1	1	4	1	1.5
26		1	2	2	1	2	4	< .18
32		4	4	4	64	32	4	3
45		1	16	1	8	16	1	< .18
62		4	8	1	>64	>32	1	3
96		2	4	1	8	16	1	< .18
99		1	4	1	8	16	1	.375
100		2	4	1	8	16	1	.375
15	Unidentified coliform	1	1	1	1	4	1	3
67		4	4	2	>64	16	8	< .18

Cat.No.	Isolate	M.I.C. in $\mu g ml^{-1}$ Ap	Sm	Tc	Su	Cx	Cm	Tm

APPENDIX .4 (a) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM ABOVE THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SA10		1	2	128	32	2	.5
16		1	2	256	32	2	32
19		1	2	256	32	2	32
27		1	1	128	32	2	.5
37		1	1	128	32	2	.5
48		1	2	256	64	2	.5
57	S.faecalis	1	1	128	32	2	.5
58	var.	1	1	128	32	1	.5
64	faecalis	1	1	128	16	2	.5
79		2	4	256	32	4	.25
86		1	1	256	16	2	.5
89		.5	1	256	16	2	.5
92		1	2	256	16	1	64
95		1	1	128	16	1	.5
96		1	2	256	32	.5	1
98		1	2	128	16	1	32
13		1	2	256	32	1	64
25	S.faecalis var.	1	1	128	32	2	.5
26	liquefaciens	1	1	128	32	2	.5
28		1	1	128	32	2	.5

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SA 4	S.faecalis var.zymogenes	1	1	256	32	2	.5
SA 3		2	2	256	64	2	.25
6		.5	1	256	64	1	.25
9		4	4	>1000	32	4	.5
12		2	4	256	64	2	.5
14		2	2	64	16	1	.5
18		1	1	128	32	.5	.25
21	S. faecium	2	2	256	32	2	.25
24		1	2	256	32	2	.25
33		2	2	256	32	2	.25
36		1	2	128	32	2	.25
38		1	.5	512	64	.125	.25
39		.25	.25	512	64	2	.25
42		.5	.5	512	32	2	.25
45		.5	.5	512	32	2	.25
54		.25	.5	256	64	1	.25
70		2	4	256	64	2	.25
71		2	4	256	64	4	.25
73		2	4	256	64	4	.5
74		2	4	256	32	4	.5
76		2	4	256	32	4	.25

APPENDIX 4 (a) (Contd.) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM ABOVE THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SA77	S. faecium	2	4	256	64	4	.25
78		1	1	64	8	.125	.5
82		.5	4	256	128	2	.25
84		1	1	64	16	.125	.5
85		2	4	256	16	4	.25
88		2	4	512	64	4	.25
94		.25	.5	512	64	.5	.25
99		2	2	128	16	.5	.5
7	S. faecium var. casseliflavus	2	2	64	16	1	.5
11		.5	.5	16	32	.5	.5
15		.5	.5	256	32	.5	.5
17		2	2	128	16	.5	.5
22		.5	.25	64	8	2	.25
23		1	2	256	32	.5	.5
29		.5	.5	16	32	.5	.5
30		.5	1	16	32	.5	.5
31		1	2	256	32	.5	.5
32		.5	.5	16	32	.5	.5
34		1	2	256	32	.5	.5
35		1	2	16	32	1	.5
40		.5	.5	256	32	.25	.5

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SA41	S. faecium var. casseliflavus	2	2	16	32	.125	.5
43		1	1	512	64	.03	.5
44		1	2	16	32	.125	.5
46		2	2	16	32	.125	.5
47		2	2	256	32	.5	.5
51		.5	1	16	32	.25	.5
56		1	2	16	32	1	.5
59		.5	.5	256	32	.5	.5
60		.5	.5	16	32	.5	.5
61		.5	.5	256	8	4	.5
62		2	2	256	32	1	.5
66		2	2	256	32	1	.5
68		.25	.125	64	8	4	1
72		2	2	256	32	.125	.5
75		.5	1	256	32	.5	.5
80		.5	.5	256	64	.125	.5
81		1	.5	16	64	.5	.5
83		1	1	128	16	.5	.5
87		1	1	256	64	.5	.5
90		.5	.5	256	32	.5	.5
93		2	2	256	32	.5	.25
100		.5	.5	256	32	.5	.5

APPENDIX 4 (a) (Contd.) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM ABOVE THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SA 1	S. durans	2	2	128	32	.5	.25
8		.25	1	256	64	.25	.5
20		1	1	256	64	.125	.5
49		.5	.5	512	32	.03	.5
52		.5	2	256	64	.03	.5
53		.5	1	512	64	.125	.5
55		.03	.03	256	32	.03	.5
63		1	1	512	64	.125	.5
65		.5	1	512	64	.03	.5
67		1	1	512	64	.125	.5
69		.5	1	512	64	.125	.25
91		.5	2	512	64	.03	64
2	Unidentified gp D	2	2	128	32	.5	.25
5	Streptococci	2	2	128	16	.5	.5

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc

APPENDIX .4 (b) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM BELOW THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SB13		2	2	256	32	.5	.5
14		2	2	256	16	1	.5
16		2	2	256	16	.03	.5
17		1	2	256	16	1	.5
18		1	2	256	16	1	.5
19		1	2	256	16	.5	.5
20		1	2	256	16	2	.5
22	S. faecalis var.	1	1	256	16	.5	.5
23	faecalis	1	2	256	32	2	.5
25		1	2	256	16	.5	.5
27		1	2	256	16	.5	.5
28		1	2	256	16	.5	.5
32		2	2	256	32	2	64
35		1	2	256	8	1	.5
38		1	2	256	16	.5	64
39		1	2	256	16	.125	.5
40		1	2	256	16	1	.5
44		1	2	256	16	2	.5
46		1	2	256	16	1	.5
47		1	2	256	16	1	.5
53		1	2	256	16	1	1
56		1	2	256	32	1	.5

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SB71		1	4	512	16	1	.5
73		1	2	256	16	1	.5
74		1	2	512	16	1	64
76		1	4	256	16	1	.5
77		1	2	256	16	1	64
79		1	2	256	16	2	64
80	S. faecalis var.	1	2	256	16	2	64
81	faecalis	1	2	256	16	128 ~~1000~~	.5
83		1	2	512	16	.125	.5
84		1	1	256	16	128	.5
85		1	2	512	16	.25	.5
86		1	2	512	16	.125	.5
94		1	2	>1000	16	>1000	512
97		1	2	256	8	.5	.5
98		1	2	256	8	.5	.5
99		1	2	256	32	1	.5
100		1	1	512	16	.125	.5
78		1	2	256	16	1	.5
90	S. faecalis var.	1	2	512	32	.5	.5
93	liquefaciens	1	1	512	16	.5	.5

APPENDIX 4 (b) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM BELOW THE SEWER OUTFALL (Contd.)

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SB37	S. faecalis var.	1	2	256	16	1	.5
52	zymogenes	1	2	256	16	2	128
2		1	4	256	64	2	.25
3		.5	1	256	64	2	.25
4		2	4	256	64	1	.25
5		1	4	256	64	1	.25
6		1	4	256	64	2	.25
11		2	4	256	64	2	.25
15		.5	1	256	64	4	.25
21		.25	.5	256	64	03	.25
30	S. faecium	2	4	256	32	03	.25
33		.5	1	256	128	.5	.25
36		.5	2	256	32	.5	.25
45		.25	2	256	64	.25	.25
48		.25	2	256	64	.25	.25
51		.25	.5	256	16	2	.25
55		.5	2	256	64	2	.25
58		.5	2	256	64	2	.25
60		1	4	256	64	2	.25
62		1	1	64	8	03	64
63		.25	.25	256	64	03	.25
64		1	4	256	64	1	.25

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SB65		1	2	64	8	03	64
68		1	1	64	8	03	64
69		16	32	1000	64	.5	64
72	S. faecium	1	4	256	64	1	.25
88		1	1	64	8	03	64
91		0.5	2	256	16	03	.5
31		.5	.25	32	4	4	.5
34		.5	.5	32	4	2	.5
41	S. faecium var.	1	2	64	8	03	64
42	casseliflavus	.5	2	256	64	2	.25
75		1	1	256	64	2	.25
82		1	1	64	8	03	64
92		.25	2	256	64	1	.5
1		1	2	512	256	03	.25
7		1	2	256	16	03	.5
8	S. durans	1	1	512	256	03	.25
9		1	2	256	16	03	.5
10		1	2	256	16	03	.5
12		2	2	512	256	03	.25
26		.5	2	256	16	03	.25
29		.5	2	256	16	03	.25
50		2	2	512	256	03	.25

APPENDIX .4 (b) (Contd.) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM BELOW THE SEWER OUTFALL

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
SB54	S. durans	1	2	512	64	.03	.25
57		1	2	512	64	.03	.25
59		1	2	512	256	.03	.25
61		.25	4	256	64	.03	.5
70		.5	1	512	64	.03	.25
87		1	4	256	16	.25	.25
89		1	2	512	256	.03	.25
95		1	2	1000	128	.03	.5
43	S. bovis	.125	.06	256	32	.03	.25
96		.5	2	256	16	.03	.5
67	S. equinus	.06	.03	256	16	.125	64
24	Unidentified grp. D. streptococci	1	4	512	256	.03	.25
49		1	1	64	4	.5	64
66		16	32	>1000	64	0.5	64

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc

APPENDIX 4 (c) M. I. C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM CLINICAL SOURCES

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
K 1	S. faecalis var. faecalis	2	2	256	16	1	0.5
2		2	2	>1000	16	1	512
3		2	2	512	32	4	1
4		2	2	256	16	2	64
5		2	2	512	16	1	1
6		2	2	256	16	1	1
9		2	2	256	16	.03	1
10		2	2	512	64	2	1
11		1	2	256	16	8	64
12		2	2	256	16	2	32
14		1	1	256	16	1	1
15		2	1	64	16	.03	1
17		2	2	256	32	.03	1
21		2	2	512	32	4	1
22		2	2	256	16	8	256
24		2	2	256	16	2	1
25		2	2	512	32	.03	1
26		2	2	256	16	2	1
28		1	2	256	16	8	1
29		2	2	>1000	16	1	64
30		2	2	>1000	16	1	64
32		2	2	256	32	2	.5

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
K34	S. faecalis var. faecalis	2	2	256	16	.5	1
36		2	2	>1000	16	4	256
37		2	2	512	16	1	64
38		2	2	512	16	2	1
39		2	2	256	16	>1000	64
40		2	2	256	32	2	1
41		2	2	256	32	2	32
43		2	2	>1000	32	2	256
44		2	2	256	32	4	32
45		2	2	512	32	4	64
47		2	2	256	32	4	64
48		2	2	256	32	1	64
50		2	1	256	32	1	1
52		2	2	512	32	4	64
53		2	2	>1000	16	4	64
55		1	2	>1000	32	>1000	64
59		2	2	256	32	2	128
62		2	2	256	16	4	1
64		8	8	256	32	.5	1
65		2	2	256	16	2	64
67		2	2	256	32	8	1
69		2	2	256	16	.5	1

APPENDIX .4 (c) (Contd.) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM CLINICAL SOURCES

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
K70	S. faecalis var. faecalis	1	1	256	16	.5	1
71		2	2	256	32	4	1
72		1	2	256	16	2	1
73		2	2	>1000	16	1	64
74		2	2	256	16	2	1
76		1	2	512	16	8	128
77		2	2	>1000	16	1	1
79		2	2	>1000	16	1	64
81		2	2	256	16	2	1
82		2	2	>1000	32	8	64
83		2	2	>1000	16	2	128
84		2	2	256	16	2	1
85		2	2	>1000	16	1	256
89		2	2	256	16	1	64
91		2	2	256	16	1	64
93		2	2	256	16	1	64
94		2	2	>1000	16	1	64
96		1	2	256	16	1	1
97		2	2	512	32	8	64
99		2	2	256	16	8	1

Cat.No.	Isolate	M.I.C. in μgml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
K16	S. faecalis var. liquefaciens	2	2	512	32	1	1
31		2	2	256	32	2	.5
42		2	2	256	32	4	64
56		2	2	>1000	32	4	64
57		2	2	512	32	1	64
60		1	2	>1000	16	>1000	512
61		2	2	256	16	.03	1
63		2	2	256	16	.5	1
66		2	2	256	16	2	1
78		2	4	256	16	16	128
86		1	2	256	32	1	1
88		2	2	>1000	16	>1000	512
90		2	2	256	16	2	128
92		2	2	256	16	.03	1
7	S. faecalis var. zymogenes	2	2	512	16	4	64
8		2	2	>1000	16	2	256
13		2	2	512	32	1	1
18		2	2	256	32	1	64
19		2	2	256	32	2	32
20		2	2	256	16	1	64
27		2	2	256	32	1	64
35		2	2	>1000	16	2	512

APPENDIX 4 (c) (Contd.) M.I.C. LEVELS OF GROUP D STREPTOCOCCI ISOLATES FROM CLINICAL SOURCES

Cat.No.	Isolate	M.I.C. in μ gml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc
K49		1	1	256	32	1	64
68		2	2	256	16	.25	1
75	S. faecalis var.	2	2	256	32	1	1
80	zymogenes	2	2	128	32	.25	1
87		2	2	1000	16	1000	512
98		2	2	256	8	8	32
100		2	2	256	16	8	32
46	S. faecium	16	32	1000	64	1000	64
54		2	2		32	4	64
23	S. faecium var. casseliflavus	1	.5	64	8	4	1
33	S. durans	1	2	512	32	.5	32
51		1	2	256	16	8	64
95		1	1	>1000	8	1	64
58	Unidentified grp. D streptococci	2	2	256	32	1	128

Cat.No.	Isolate	M.I.C. in μ gml^{-1} of: Ap	Pn	Sm	Gm	Em	Tc

6. BIBLIOGRAPHY

BIBLIOGRAPHY

ABRAHAM, E.P. and CHAIN, E. 1940. Nature 146, 837.

ABSHIRE, R.L. 1977. Appl. Environ. Microbiol. 33, 1149.

ACHTMAN, M. and HELMUTH, R. 1974. In Microbiology - 1974 p.95 ed. SCHLESSINGER, D. Am. Soc. Microbiol., Washington, D.C. 1975.

ALLEN, L.A., PASLEY, S. M. and PEARCE, M.A.F. 1952. J. Gen. Microbiol. 7, 36.

ANDERSON, E.S. 1968. Ann. Rev. Microbiol. 22, 131.

ANDERSON, J.D., ADAMS, M.A., BARRINGTON, J.C., SPENCE, W.N. and SHEPHERD, C.A. 1976. Antimicrob. Ag. Chemother 10, 872.

ANDREWS, J., BYWATER, M.J., EMMERSON, A.M., KEANE, C., REEVES, D.S. and WISE, R. 1975. Proc. 9th Int. Cong. Chemother. London Chemother 3, 271.

ATOR, L.L. and STARZYK, M.J. 1976. Microbios. 16, 91.

AVERY, O.T., McLEOD, C.M. and McCARTY, M. 1944. J. Exp. Med. 79, 137.

AYLIFFE, G.A.J., GREEN, W., LIVINGSTON, R. and LOWBURY, E.J.L. 1977. J. Clin. Pathol. 30, 40.

BALOWS, A. 1977. J. Antimicrob. Chemother 3 Suppl. C,3.

BARBOUR, S.D. 1967. J. Mol. Biol. 28, 373.

BARNES, E.M. 1956. J. Gen. Microbiol. 14, 57.

BARRIE, J.D. 1980. Personal Communication.

BARRITT, M.M. 1936. J. Path. Bact. 42, 441.

BARTLEY, C.H. and SLANETZ, L.W. 1960. Am. J. Pub. Hlth. 50, 1545.

BENNETT, P.M. and RICHMOND, M.H. 1979. In: The Bacteria A treatise on Structure and Function. Vol. 6. Bacterial Diversity p1, eds. ORNSTON, L.N. and SOKATCH, J.R. Academic Press, New York.

BERG, G. 1966. ed. Transmission of viruses by the water route. Interscience Publishers, New York.

BERGEY, D.H. 1974. Bergey's Manual of Determinative Bacteriology 8th edn. eds. BUCHANAN, R.E. and GIBBONS, N.E. Williams and Wilkins Co. Baltimore.

BIGGER, J.W. and NELSON, J.H. 1941. J. Path. Bact. 53, 159.

BIGGER, J.W. and NELSON, J.H. 1943. J. Path. Bact. 55, 321.

BISS ONETTE, G.K., JEZESKI, J.J., McFETERS, G.A. and STUART, D.G. 1975. Appl. Microbiol. 29, 186.

BLAZEVIC, D.J. and EDERER, G.M. 1975. Principles of biochemical tests in diagnostic microbiology. John Wiley and Sons, New York.

BONDE, G.J. 1962. Bacterial indicators of water pollution. Teknisk Forlag.

BARDSLEY, D.A. 1934. J. Hyg. Camb. 34, 38.

BRIGGS, C.A.E., WILLINGALE, J.M., BRAUDE, R. and MITCHELL, K.G. 1954. Vet. Rec. 66, 241.

BRODA, P. 1979. Plasmids. W.H. Freeman and Co., Oxford.

BRYANT, M.P. and BURKEY, L.A. 1953. J. Dairy Sci. 36, 205.

BUDD, W. 1856. Lancet ii 618, 694.

BUDD, W. 1873. Typhoid Fever: Its nature, mode of spreading and prevention. Longmans Green. London. Republished by the American Public Health Association. New York. 1931. pp.184.

BURMAN, N.P., STEVENS, J.K. and EVANS, A.W. 1978. In: Streptococci p.335 eds. SKINNER, F.A. and QUESNEL, L.B. Academic Press, London.

BUSHBY, S.R.M. 1973. In: Trimethoprim - sulphamethoxazole microbiological, pharmacological and clinical consideration. p.11 eds. FINLAND, M. and KASS, E.H. Univ. Chicago Press, Chicago.

BUTTIAUX, R. 1958. Ann. Inst. Pasteur. Lille 94, 778.

BUTTIAUX, R. and MOSSEL, D.A.A. 1961. Appl. Bact. 24, 353.

CARNER, M.J. and KOGUT, M. 1980. FEMS Microbiology Letters 9, 115.

CHAKRABARTY, A.M. 1976. Ann. Rev. Genet. 10, 7.

CHENLETT, E.T. 1979. Environmental Protection. McGraw-Hill series in water resources and environmental engineering. McGraw-Hill Book Co.

CHRISTENSEN, P., KAHLMETER, G., JONSSON, S. and KRONVALL, G. 1973. Infect. Immun. 7, 881.

CLEWELL, D.B. and HELINSKI, D.R. 1970. Biochem. 9, 4428.

CLEWELL, D.B., YAGI, Y., DUNNY, G.M. and SCHULTZ, S.K. 1974. J. Bact. 117, 283.

CLOSS, O. and DIGRANES, A. 1971. Acta Pathol. Microbiol. Scan. Sect. B 79, 673.

CLOWES, R.C. 1971. Bact. Rev. 36, 361.

COHEN, B. 1922. J. Bact. 7, 183.

COLES, H.G. and SIMPSON, N.W. 1958. Proc. Soc. Wat. Treat. Exam. 7, 173.

COLMAN, G. 1968. J. Gen. Microbiol. 50, 149.

COOPER, K.E. and RAMADAN, F.M. 1955. J. Gen. Microbiol. 12, 180.

COWAN, T.S. 1974. Cowan and Steel's Manual for the identification of medical bacteria. 2nd edn. Camb. Univ. Press.

COWLES, P.B. 1931. J. Bact. 21, 161.

COZZARELLI, N.R., KELLY, R.B. and KORNBERG, A. 1968. Proc. Natl. Acad. Sci. U.S.A. 60, 992.

CROSA, J.H., BRENNER, D.J. and FALKOW, S. 1973. J. Bact. 115, 904.

CRUIKSHANK, R., DUGUID, J.P., MARMION, B.P. and SWAIN, R.H.A. 1975. Medical Microbiology. Churchill-Livingstone, Edinburgh.

DATTA, N. 1974. In: Microbiology-1974. p.9 ed. SCHLESSINGER, D.,Am. Soc. Microbiol. Washington, D.C. 1975.

DATTA, N. 1975. In: Microbial drug resistance. p.83 eds. MITSUHASHI, S. and HASHIMOTO, H. University Park Press.

DAVENPORT, C.V. et al. 1976. Quoted from SHAH, A. 1980. B.Sc. Thesis University of St. Andrews.

DAVIS, B.D. 1950. J. Bact. 60, 507.

DEIBEL, R.H. 1964. Bacteriol. Rev. 28, 330.

DEIBEL, R.H., LAKE, D.E. and NIVEN, C.F. 1963. J. Bact. 86, 1275.

D.H.S.S. (Welsh Office), 1969. Reports on public health and medical subjects No.71. The bacteriological examination of water supplies. H.M.S.O., London.

D'HERELLE, F. 1917. C.R. Acad. Sci. 165, 373.

D'HERELLE, F. 1921. Le bacteriophage: son rôle dans l'immunite. Mason, Paris. English translation: Williams and Wilkins, Baltimore and London.

DIBLE, J.H. 1921. J. Path. Bact. 24, 3.

DICKINSON, A.B. and MOCQUOT, G. 1961. J. Appl. Bact. 24, 252.

DONNELLY, S.L. and HARTMAN, P.A. 1978. Appl. Environ. Microbiol. 35, 576.

DRASAR, B.S. and HILL, M.J. 1974. Human Intestinal Flora. Academic Press, London.

DUFOUR, A.P. and CABELLI, V.J. 1975. Appl. Microbiol. 29, 826.

DUTKA, B.J. 1973. J. Environ. Hlth. 36, 39.

80/778/EEC. 1980. The quality of water for human consumption.

ELLIOTT, S.D. 1966. J. Hyg. Camb. 64, 205.

EL- SOLH, N., BOUANCHAUD, D.H., HORODNICEANU, T., ROUSSEL, A.T. and CHABBERT, Y.A. 1978. Antimicrob. Ag. Chemother 14, 19.

ENGEL, H.W.B., SOEDIRMAN, N., ROST, J.A., van LEEUWEN, W.J. and van EMBDEN, J.D.A. 1980. J. Bact. 142, 407.

ERICSSON, H.M. and SHERRIS, J.C. 1971. Acta Pathol. Microbiol. Scan. Sec. B Suppl. 217.

EVISON, L.M. and JAMES, A. 1973. J. Appl. Bact. 36,109.

EVISON, L.M. and JAMES, A. 1975. Prog. Wat. Tech. 7, 57.

FACKLAM, R.R. 1972. Appl. Microbiol. 23, 1131.

FACKLAM, R.R. and MOODY, M.D. 1970. Appl. Microbiol. 20, 245.

FACKLAM, R.R., PADULA, J.F., THACKER, L.G., WORTHAM, E.C. and SCONYERS, B.J. 1974. Appl. Microbiol. 27, 107.

FAIR, J.F. and MORRISON, J.M. 1967. Wat. Res. Resources 3, 799.

FALKOW, S. 1975. Infectious multiple drug resistance. Pion Ltd., London.

FALKOW, S., GUERRY, P., HEDGES, R.W. and DATTA, N. 1974. J. Gen. Microbiol. 85, 65.

FASS, R.J. and PRIOR, R.B. 1978. Curr. Therapeut. Res. 24, 352.

FEACHEM, R. 1975. Wat. Res. 9, 689. Pergamon Press.

FENLON, D. 1981. Proc. the Eastern regional medical/veterinary Liaison Committee, Dundee.

FINEGOLD, S.M. 1977. Anaerobic bacteria in human disease. Academic Press, New York.

FONTAINE, T.D. and HOADLEY, A.W. 1976. Hlth. Lab. Sci. 19, 239.

FOOD and DRUG ADMINISTRATION 1972. Fed. Register 37, 20525.

FOOD and DRUG ADMINISTRATION 1973. Fed. Register 38, 2576.

FREIFELDER, D.R. and FREIFELDER, D. 1968. J. Mol. Biol. 32, 25.

FROST, W.D. and ENGELBRECHT, M.A. 1940. The Streptococci, their description with special reference to those in milk. Willdof Book Co., Madison, Wisconsin.

FUJIOKA, R.S., HASHIMOTO, H.H., SIWAK, E.B. and YOUNG, R.H.F. 1981. Appl. Environ. Microbiol. 41, 690.

GALE, E.F., CUNDLIFFE, E., REYNOLDS, P.E., RICHMOND, M.H. and WARING, M.J. 1972. The molecular basis of antibiotic action. John Wiley and Sons, London.

GALLAGHER, T.T. and SPINO, D.F. 1968. Wat. Res. 2, 169.

GARROD, L.P., LAMBERT, H.P. and O'GRADY, F. 1973. Antibiotic and chemotherapy. 4th edn. Churchill-Livingstone, Edinburgh.

GATES, F.L. 1930. J. Gen. Physiol. 14, 31.

GELDREICH, E.E. 1966. Wat. Pollut. Contr. Res. Publ. No.WP.20.3, 122.

GELDREICH, E.E. and KENNER, B.A. 1969. J. Wat. Poll. Contr. Fed. R336.

GELDREICH, E.E., BEST, L.C., KENNER, B.A. and van DONSEL, D.J. 1968. J. Wat. Pollut. Contr. Fed. 40, 1861.

GRABOW, W.O.K. and PROZESKY, O.W. 1973. Antimicrob. Ag. Chemother 3, 175.

GRANCHER, J. and DESCHAMPS, E. 1889. Arch. Med. Exptl. Anat. Pathol. 1, 33.

GRAUDAL, H. 1951. Acta Path. et Microbiol. Scan. 31, 46.

GREEN, D.M., SCOTT, S.S. and MOWAT, D.A. 1968. J. Hyg. Camb. 66, 383.

GRIFFITH, F. 1928. J. Hyg. 27, 113.

GRINDLEY, N.D.F., HUMPHREYS, G.O. and ANDERSON, E.S. 1973. J. Bact. 115, 387.

GRINDSTED, J., SAUNDER, J.R., INGRAM, L.C., SYKES, R.B. and RICHMOND, M.H. 1972. J. Bact. 110, 529.

GROSS, K.C., HOUGHTON, M.P. and SENTERFIT, L.B. 1975. J. Clin. Microbiol. 1, 54.

GROSS, W.A., DIETZ, W.H. and COOK, R.M. 1965. J. Bact. 89, 1068.

GRUNNETT, K. and NIELSEN, B.B. 1969. Appl. Microbiol. 18, 985.

GUELIN, A. 1952. Ann. Inst. Pasteur Lille 82, 78.

GUERRY, P. and FALKOW, S. 1971. J. Bact. 107, 372.

GUERRY, P., FALKOW, S. and DATTA, N. 1974. J. Bact. 119, 144.

GUNSALUS, I.C. 1947. J. Bact. 54, 229.

GYLLENBERG, H. and NIEMELÄ, S. 1959. J. Sci. Agr. Soc. Finland 31, 94.

HAENEL, H. 1960. Zbl. Bakt. (Abt.1 Ref) 176, 305.

HAJNA, A.A. and PERRY, C.A. 1943. Am. J. Pub. Hlth. 33, 550.

HAMILTON, W. 1935. A study of the pollution of River Whangpoo as affecting its use as a source of water supply. The Mercantile Printing Co. Ltd., Shanghai.

HANNAY, C.L. and NORTON, I.L. 1947. Proc. Soc. Appl. Bact. No.1, 139.

HARTLEY, C.L. and RICHMOND, M.H. 1975. Brit. Med. J. 4, 71.

HARTMAN, P.A., REINBOLD, G.W. and SARASWAT, D.S. 1966. Int. J. Syst. Bacteriol. 16, 197.

HARTMAN, P.A., REINBOLD, G.W. and SARASWAT, D.S. 1966. In: Advances in applied microbiology. p.253 ed. UMBREIT, W.W. Vol.8. Academic Press, New York.

HAYES, W. 1968. The genetics of bacteria and their viruses. Blackwell, Oxford.

HELINSKI, D.R. 1973. Ann. Rev. Microbiol. 27, 437.

HELLER, C.L. 1954. J. Appl. Bacteriol. 17, 202.

HICKSON, F.T., ROTH, R.F. and HELINSKI, D.R. 1967. Proc. Natl. Acad. Sci. U.S.A. 58, 1731.

HIGGINS, I.J. and BURNS, R.G. 1975. The chemistry and microbiology of pollution. Academic Press, London.

HOBBS, B.C. 1961. J. Appl. Bact. 24, 340.

HOLDEMAN, L.Y., CATO, E.P. and MOORE, W.E.C. 1977. Anaerobe Laboratory Manual 4th edn. Anaerobe Laboratory, Virginia Polytechnic Institute, and State University, Blacksburg, Virginia.

HOLDEN, W.S. 1970. ed. Water treatment and examination. J. and H. Churchill, London. p.243.

HOSPITAL DOCTOR, 1981. Hospital Doctor 6.7, 6.

HOTCHKISS, R.D. and GARBOR, M. 1970. Ann. Rev. Genet. 4, 193.

HOUSTON, A.C. 1900. Ann. Rep. Local Govt. Board. Report of Medical Officer 1889-1900. L.C.C., 458.

HOUSTON, A.C. 1913. Studies in water supply. McMillan & Co.

HUGHES, C. and MEYNELL, G.C. 1974. Lancet 1975 ii, 451.

INTERNATIONAL CODE OF NOMENCLATURE OF BACTERIA AND VIRUSES 1948. J. Bact. 55, 287.

INTERNATIONAL CODE OF NOMENCLATURE OF BACTERIA AND VIRUSES 1948. Ames Iowa, Iowa State University Press.

JACOB, A.E. and HOBBS, S.J. 1974. J. Bact. 117, 360.

JACOB, A.E., DOUGLAS, G.J. and HOBBS, S.J. 1975. J. Bact. 121, 863.

JEFFRIES, C.D., HOLTMAN, D.F. and GUSE, D.G. 1957. J. Bact. 73, 590.

JONES, D. 1978. In: Streptococci. p.1 eds. SKINNER, F.A. and QUESNEL, L.B. Academic Press, London.

JONES, D., DEIBEL, R.H. and NIVEN, Jr. C.F. 1963. J. Bact. 86, 171.

JONES, P.W., RENNISON, L.M., LEWIN, V.H. and REDHEAD, D.L. 1980. J. Hyg. Camb. 84, 47.

KARLINSKI, J. 1889. Centr. Bakteriol. Abt. 25, 26.

KEICH, W.J. and LEE, J.S. 1978. Appl. Environ. Microbiol. 36, 450.

KENNER, B.A., CLARK, H.F. and KABLER, P.W. 1960. Am. J. Pub. Hlth. 50, 1555.

KENNER, B.A., CLARK, H.F. and KABLER, P.W. 1961. Appl. Microbiol. 9, 15.

KIRBY, R. 1976. Ph.D. Thesis. University of East Anglia, England.

KJELLANDER, J. 1960. Acta Path. et Microbiol. Scan. Suppl. 136, 48, 1.

KONINGS, W.N. and VELDKAMP, H. 1980. In: Contemporary microbial ecology. p.161 eds. ELLWOOD, D.C., HEDGER, J.N., LATHMAN, M.J., LYNCH, J.M. and SLATER, J.H. Academic Press, London.

KOOL, A.J. and NIJKAMP, H.J.J. 1974. J. Bact. 120, 569.

LANE-CLAYPTON, J.E. 1909. Quoted from MRC System of Bacteriology, Vol.1, 1929.

LANTOS, J., VARGA, I. and IVANOVICS, G. 1960. Acta Microbiol. Acad. Sci. Hung. 7, 31.

LEDERBERG, J. and LEDERBERG, E.M. 1952. J. Bact. 63, 399.

LENNETTE, E.H., SPAULDING, E.H. and TRUANT, J.P. 1974. eds. Manual of Clinical Microbiology. 2nd edn. Am. Soc. Microbiol. Washington, D.C.

LINDIN-JANSON, G., FABEN, E., JODAL, U., KAIJSER, B. and LINCOLN, K. 1977. J. Med. Microbiol. 10, 229.

LINTON, K.B., RICHMOND, M.H., BEVAN, R. and GILLESPIE, W.A. 1974. J. Gen. Microbiol. 7, 91.

LITSKY, W., MALLMANN, W.L. and FIFIELD, C.W. 1953. Am. J. Pub. Hlth. 43, 873.

LOVELL, D.J. and BIBEL, D.J. 1977. J. Clin. Microbiol. 5, 225.

LOWE, G.H. 1962. J. Med. Lab. Technol. 19, 21.

MACKIE, J. 1913. J. Path. Bact. 18, 137.

MALLMANN, W.L. 1940. Sew. Works J. 12, 875.

MALLMANN, W.L. and SELIGMANN, Jr. E.B. 1950. Am. J. Pub. Hlth. 40, 286.

MARA, D.D. 1974. Bacteriology for Sanitary Engineers. p.98. Churchill-Livingstone, Edinburgh.

MARDER, H.P. and KAYSER, F.H. Antimicrob. Ag. Chemother 12, 261.

NISIOKA, T., MITANI, M. and CLOWES, R.C. 1970. J. Bact. 103, 166.

NOBLE, C.J. 1978. J. Clin. Pathol. 31, 1182.

NOVICK, R.P. 1969. Bact. Rev. 33, 210.

NOVICK, R.P. and BRODSKY, R. 1972. J. Mol. Biol. 68, 285.

NOVICK, R.P. and RICHMOND, M.H. 1965. J. Bact. 90, 467.

NOVICK, R.P., CLOWES, R.C., COHEN, S.N., CURTISS, R.III., DATTA, N. and FALKOW, S. 1976. Bact. Rev. 40, 168.

OLSEN, R.H. and SHIPLEY, P. 1973. J. Bact. 113, 772.

OPARA, A.A. 1978. Ph. D. Thesis. University of Dundee.

ORLA-JENSEN, S. 1919. The lactic acid bacteria. Adv. Fed. Host and Son, Copenhagen.

PARKER, M.T. 1978. In: The Streptococci. p.71 eds. SKINNER, F.A. and QUESNEL, L.P. Academic Press, London.

PARKER, M.T. and BALL, L.C. 1976. J. Med. Microbiol. 9, 275.

PARR, L.W. 1938. J. Bact. 36, 1.

PAVLOVA, M.T., BREZENSKI, M.S. and LITSKY, W. 1972. Hlth. Lab. Sci. 9, 289.

PHILLIPS, I. 1975. Personal Communication.

PHILLIPS, I. 1981. Oxoid Vol.2 No.1 Oxoid Ltd.

POWNALL, M. 1935. Brit. J. Exp. Path. 16, 155.

PUBLIC HEALTH LABORATORY SCIENCE WATER SUB-COMMITTEE 1952. J. Hyg. Camb. 50, 107.

RAIBAUD, P., CAULETT, M., GALPIN, J.V. and MOCQUOT, G. 1961. J. Appl. Bact. 24, 285.

RESNICK, G. and LEVIN, M.A. 1977. Proc. Am. Soc. Microbiol.

RHEINHEIMER, G. 1974. Aquatic Microbiology. J. Wiley and Sons.

RICHMOND, M.H. 1972. J. Appl. Bact. 35, 155.

RICHMOND, M.H. and LINTON, K.B. 1980. J. Antimicrob. Ther. 6, 33.

RIVA, S., FIETTA, A., BERTI, M., SIVESTRI, L.G. and RUMEO, E. 1973. Antimicrob. Ag. Chemother 3, 456.

ROOP, D.R., MUNDT, J.O. and RIGGSBY, W.S. 1974. Int. J. Syst. Bacteriol. 24, 330.

ROSENBLATT, J.E., FALLEN, A. and FINEGOLD, S.M. 1973. Appl. Microbiol. 25, 77.

ROUND TABLE DISCUSSION. SECOND INTERNATIONAL SYMPOSIUM ON MICROBIAL ECOLOGY 1980. Warwick University, Coventry.

RUPERT, C.S. 1964. In: Photophysiology. p.283. ed. GIESE, A.C. Academic Press, New York.

SABBAJ, J., SUTTER, V.L. and FINEGOLD, S.M. 1971. Appl. Microbiol. 22, 1008.

SAVAGE, D.C. 1977. Ann. Rev. Microbiol. 31, 107.

SCHREMPF, H., BUJARD, H., HOPWOOD, D.A. and GOEBEL, W. 1975. J. Bact. 121, 416.

SEELEMAN, M. 1954. Biologie de Streptokokken. Hans Carl. Nurenberg.

SELIGMANN, R. and REITLER, R. 1965. J. Am. Wat. Wks. Assn. 57, 33.

SHAH, P.P., DALIUS, J.B., ROBSON, H.G. and CONTERATO, J.P. 1979 Antimicrob. Ag. Chemother 15, 346.

SHATTOCK, P.M.F. 1949. J. Gen. Microbiol. 3, 80.

SHATTOCK, P.M.F. 1955. Ann. Inst. Pasteur Lille 7, 95.

SHATTOCK, P.M.F. 1962. In: Chemical and biological hazards in food. p.303. eds. KRAFT, J.C., SYNDER, A.A. and WALKER, H.W. Ames Iowa State - University Press.

SHAW, C. and CLARKE, P.H. 1955. J. Gen. Microbiol. 13, 155.

SHERMAN, J.M. 1937. Bacteriol. Rev. 1, 3.

SHERMAN, J.M. 1938. J. Bact. 35, 81.

SHERMAN, J.M. and WING, H.U. 1935. J. Dairy Sci. 18, 657.

SHERMAN, J.M. and WING, H.U. 1937. J. Dairy Sci. 20, 165.

SHERRIS, J.C. 1974. In: Manual of Clinical Microbiology. p.407. ed LENNETTE, E.H., SPAULDING, E.H. and TRUANT, J.P. 2nd edn. Am. Soc. Microbiol. Washington, D.C.

SIBONI, K. 1980. Acta Pathol. Microbiol. Scad. Sect. B 88, 189.

SKADHAUGE, K. 1950. Studies on the enterococci with special reference to the serological properties. Einar Munksgaards. Forlag, Copenhagen.

SKINNER, F.A. and CARR, J.G. 1974. eds. The normal microbial flora of man. Academic Press, London.

SLANETZ, L.W. and BARTLEY, C.H. 1957. J. Bact. 74, 591.

SLANETZ, L.W., BENT, D. and BARTLEY, C.H. 1955. Pub. Hlth. Rep. 70, 67.

SLOCOMBE, B., EDMONDSON, R.A. and SUTHERLAND, R. 1975. In: Drug inactivating enzymes and antibiotic resistance. p.76. eds. MITSUHASHI, S., ROSIVAL, L. and KRÖMERY, V. Avicenum. Chezchoslavak Med. Press, Prague.

SMITH, H.W. and CRABB, W.E. 1961. J. Path. Bact. 82, 53.

SMITH, D.G. and SHATTOCK, P.M.F. 1962. J. Gen. Microbiol. 29, 731.

SMITH, D.H., HUMPHREYS, G.O. and ANDERSON, E.S. 1974. Mol. Gen. Genet. 129, 229.

SNEATH, P.H.A. and SOKEL, R.R. 1973. Numerical Taxonomy. W.H. Freeman, San Francisco.

SNOW, J. 1855. On the mode of communication of cholera. 2nd edn. John Churchill, London.

SPENCER, R. 1963. In: Symposium on Marine Microbiology. p.350 ed. OPPENHEIMER, C.H. Springfield III Thomas.

STANDARD METHODS 1975. Standard methods for the examination of water and waste water. 14th edn. Am. Pub. Hlth. Assn. Washington.

STANDIFORD, H.D., DeMAINE, J.B. and KIRBY, W.M.M. 1970. Arch. Int. Med. 126, 255.

STURTEVANT, A.B. and FEARY, T.W. 1969. Appl. Microbiol. 28, 1086.

TABAQCHALI, S. 1974. In: Infection with non-sporing anaerobic bacteria. p.59. eds. PHILLIPS, I. and SUSSMAN, M. Churchill-Livingstone, Edinburgh.

TAYLOR, C.B. 1940. J. Hyg. Camb. 40, 616.

TAYLOR, E.B. and BURMAN, N.P. 1964. J. Appl. Bact. 27, 294.

TECHNICAL REPORT TR71, 1978. Wat. Res. Center Marlow, Bucks.

THEN, R. and ANGEHRN, P. 1973. In: Trimethoprim-Sulphamethoxazole. microbiological, pharmacological and clinical considerations. p.66 eds. FINIAND, M. and KASS, E.H. Univ. Chicago Press, Chicago.

THIERCELIN, M.E. 1899. Compt. Rend. Soc. Biol. 5, 269.

THOMSON, S. 1955. J. Hyg. Camb. 53, 217.

THORNBERRY, C., BAKER, C.N. and FACKIAM, R.R. 1974. Antimicrob. Ag. Chemother. 5, 228.

THORNLEY, M.J. 1960. J. Appl. Bact. 23, 37.

TOAIA, P., McDONALD, A., WILCOX, C. and FINIAND, M. 1969. Am. J. Med. Sci. 258, 416.

THRESH, J.C., BEALE, J.F. and SUCKLING, E.V. 1958. Examination of waters and water supplies. 7th edn. E. Windle Taylor, ed. London Churchill.

TWORT, F.W. 1915. Lancet ii, 1241.

UPDYKE, E.L. 1957. Pub. Hlth. Lab. 15, 78.

VAN DONSEL, D.J., GELDREICH, E.E. and CLARKE, N.A. 1967. Appl. Microbiol. 15, 1363.

VAN EMBDEN, J.D.A., ENGEL, H.W.B. and VAN KLINGEREN, 1977. Antimicrob. Ag. Chemother. 11, 925.

VAPNEK, D., LIPMAN, M.B. and RUPP, W.D. 1971. J. Bact. 108, 508.

WAITKINS, S. 1978. J. Clin. Pathol. 31, 692.

WAITKINS, S.A., BALL, L.C. and FRASER, C.A. 1980. J. Clin. Pathol. 33, 47.

WARREN, E., SYNDER, R.J., THOMPSON, C.O. and WASHINGTON III, J.A. 1972. Mayo Clinic Proc. 47, 34.

WATANABE, T. 1963. Bact. Rev. 27, 84.

WATKINS, J. and SLEATH, K.P. 1981. J. Appl. Bact. 50, 1.

WEIL-KORSTANGE, J.A.A. van der and WINKLER, K.C. 1975. J. Med. Microbiol. 8, 491.

WEINER, M. 1977. J. Clin. Microbiol. 6, 536.

WHIPPLE, G.C. and MAYER, A. 1906. J. Inf. Dis. Suppl. 2, 76.

WHITTENBURY, R. 1965. J. Gen. Microbiol. 38, 297.

WIKOWSKE, C.J., FACKLAM, R.R., WASHINGTON, J.A. and GERACI, J.E. 1974. Antimicrob. Ag. Chemother 170, 95.

WILLIAMS-SMITH, H. 1970. Nature, 228, 1286.

W.H.O. 1959. Resolution W.H.A. 12.48 Off. Rec. Wrld. Hlth. Org. 95, 42.

W.H.O. 1961. European Standards for drinking water. Wrld. Hlth. Org. Geneva.

W.H.O. 1963. International Standards for drinking water. 2nd edn. Wrld. Hlth. Org. Geneva.

W.H.O. 1967. WHO expert committee on cholera. Second Report. Wrld. Hlth. Org. Tech. Rep. Ser. 352, 3.

W.H.O. 1974. Weekly epidemiological record No.27, 229. Wrld. Hlth. Org. Geneva.

WILLSSENS, A. and BUTTIAUX, R. 1958. Ann. Inst. Past. 94, 332.

WILSON, W.J. 1931. Brit. Wat. Wks. Assn. Official Cir. 96.

WILSON, G.S. and MILES, A. 1975. Topley and Wilson's principles of bacteriology, virology and immunity. 6th edn. Edward Arnold, London.

WINDSOR, R.S. and ELLIOTT, S.D. 1975. J. Hyg. Camb. 75, 69.

MATTHEW, M. 1979. J. Antimicrob. Chemother 5, 349.

MAY and BAKER, 1979. Anaerobic agent Flagyl. May and Baker Ltd., Dagenham, England.

MEYNELL, G.G. and MEYNELL, E. 1975. Theory and practice in experimental bacteriology. 2nd edn. Cambridge University Press.

McFETERS, G.A., BISSONNETTE, G.K., JEZESKI, J.J., THOMSON, C.A. and STUART, D.G. 1974. Appl. Microbiol. 27, 823.

McGOWAN, J.E. Jr., GARNER, C., WILCOX, C. and FINLAND, M. 1974. Am. J. Med. 57, 225.

McINTOSH, J., JAMES, W.W. and LAZARUS-BARLOW, P. 1922. Brit. J. Exp. Path. 3, 138.

MEAD, G.C. 1963. Nature, London 197, 1323.

MEAD, G.C. 1965. Ph.D. Thesis, University of London.

MEAD, G.C. 1966. Proc. Soc. Wat. Treat. Exam. 15, 207.

MEDREK, T.F. and LITSKY, W. 1960. Appl. Microbiol. 8, 60.

MILLIKEN, C.E. and CLOWES, R.C. 1973. J. Bact. 113, 1026.

MOSSEL, D.A.A. 1957. Appl. Microbiol. 5, 396.

MOSSEL, D.A.A. 1959. J. Appl. Bact. 22, 184.

MOUSSA, R.S. 1965. J. Appl. Bact. 28, 466.

MULLER, G. 1964. Arch. Hyg. Bakt. 148, 321.

MUNCH-PETERSEN, E. and BOUNDY, C. 1962. Bull. Wrld. Hlth. Org. 26, 3.

MUNDT, J.O. and GRAHAM, W.F. 1968. J. Bact. 95, 2005.

NATIONAL COMMITTEE ON CLINICAL LABORATORY STANDARDS 1973. Subcommittee on antibiotic susceptibility testing.

NATIONAL COMMITTEE ON CLINICAL LABORATORY STANDARDS 1973. Performance standards for antimicrobial disc. Los Angeles.

NAVASHIN, S.N., BELJAVSKAYA, I.V., SAZYKIN, Y.O. and GRYAZNOVA, N.S. 1975. In: Drug inactivating enzymes and antibiotic resistance. p.227 eds. MITSUHASHI, S., ROSIVAL, L. and KRCMERY, V. Avicenum, Chezchoslavak Med. Press, Prague.

NAIDOO, J. and NOBLE, W.C. 1980. Exchange of plasmids between staphylococci on skin. Poster contributions Second International Symposium on Microbial ecology, Warwick.

WINSLOW, C.A.E. and HUNNEWELL, M.P. 1902. Sci. 15, 827.

WINSLOW, C.A.E. and PALMER, G.T. 1910. J. Inf. Dis. 7, 1.

WINTER, E.C. and SANDHOLZER, L.A. 1946. J. Bact. 51, 588.

ZIMMERMANN, R.A., MOELLERING, R.C. Jr., and WEINBERT, A.N. 1971. J. Bact. 105, 873.

ZASKE, S.K., DOCKINS, W.S. and McFETERS, C.A. 1980. Appl. Environ. Microbiol. 40, 386.

ZINDER, N.D. and LEDERBERG, J. 1952. J. Bact. 64, 679.